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(54) Title: BCL-2-MODIFYING FACTOR (BMF) SEQUENCES AND THEIR USE IN MODULATING APOPTOSIS

(57) Abstract: The present invention relates generally to novel molecules capable of, *inter alia*, modulating apoptosis in mammalian cells and to genetic sequences encoding same. More particularly, the present invention relates to a novel member of the Bcl-2 family of proteins, referred to herein as "Bmf", and to genetic sequences encoding same and to regulatory sequences such as a promoter sequence directing expression of Bmf. Bmf comprises a BH3 domain which facilitates interaction to pro-survival Bcl-2 family members thereby triggering apoptosis. Bmf is regarded, therefore, as a BH3-only molecule. The molecules of the present invention are useful, for example, in therapy, diagnosis, antibody generation and as a screening tool for therapeutic agents capable of modulating physiological cell death or survival and/or modulating cell cycle entry. The present invention further contemplates genetically modified animals in which one or both alleles of Bmf are mutated or partially or wholly deleted alone or in combination with a mutation in one or both alleles of another Bcl-2-type molecule such as but not limited to Bim. The genetically modified animals are useful *inter alia* in screening for agents which ameliorate the symptoms of diseases caused by defects in apoptosis or which specifically promote apoptosis of target cells.

WO 02/097094 A1

NOVEL THERAPEUTIC MOLECULES

FIELD OF THE INVENTION

The present invention relates generally to novel molecules capable of, *inter alia*, modulating apoptosis in mammalian cells and to genetic sequences encoding same. More particularly, the present invention relates to a novel member of the Bcl-2 family of proteins, referred to herein as "Bmf", and to genetic sequences encoding same and to regulatory sequences such as a promoter sequence directing expression of Bmf. Bmf comprises a BH3 domain which facilitates interaction to pro-survival Bcl-2 family members thereby triggering apoptosis. Bmf is regarded, therefore, as a BH3-only molecule. The molecules of the present invention are useful, for example, in therapy, diagnosis, antibody generation and as a screening tool for therapeutic agents capable of modulating physiological cell death or survival and/or modulating cell cycle entry. The present invention further contemplates genetically modified animals in which one or both alleles of Bmf are mutated or partially or wholly deleted alone or in combination with a mutation in one or both alleles of another Bcl-2-type molecule such as but not limited to Bim. The genetically modified animals are useful *inter alia* in screening for agents which ameliorate the symptoms of diseases caused by defects in apoptosis or which specifically promote apoptosis of target cells.

BACKGROUND OF THE INVENTION

Reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in any country.

Apoptosis, the physiologic and genetically modulated process of cell death, is of central importance for modelling tissues and maintaining homeostasis in multicellular organisms (Kerr *et al., Br. J. Cancer 26:* 239-257, 1972; Jacobson *et al., Cell 88:* 347-354, 1997). Great progress is being made towards understanding the biochemistry underlying this

intrinsic suicide program. The cellular apoptotic effector molecules include a set of cysteine proteinases, termed caspases, that degrade critical cellular substrates (Nicholson *et al., Trends Biochem. Sci. 22:* 299-306, 1997). The regulatory machinery that governs the activation of the caspases is less well understood. However, a family of proteins of which Bcl-2 is the prototypic molecule (and is referred to as the Bcl-2 family of proteins) plays a central role (Jacobson, *Curr. Biol. 7:* R277-R281, 1997; Reed, *Nature 387:* 773-776, 1997; Kroemer, *Nature Med. 3:* 614-620, 1997; Adams and Cory, *Science 281:* 1322-1326, 1998).

Bcl-2 was the first intracellular regulator of apoptosis to be identified (Vaux *et al., Nature 335:* 440-442, 1988) and high levels enhance cell survival under diverse cytotoxic conditions. Other cellular homologs, such as Bcl-x_L (Boise *et al., Cell 74:* 597-608, 1993) and Bcl-w (Gibson *et al., Oncogene 13:* 665-675, 1996), also enhance cell survival, as do more distantly related viral homologs, such as the adenovirus E1B 19K protein (White *et al., Mol. Cell. Biol. 12:* 2570-2580, 1992) and Epstein-Barr virus BHRF-1 (Henderson *et al., Proc. Natl. Acad. Sci. USA 90:* 8479-8483, 1993).

Pro-apoptotic BH3-only members of the Bcl-2 family are essential for initiation of apoptosis in species as distantly related as mice and *C. elegans* (Huang and Strasser, *Cell 103:* 839, 2000). EGL-1, the so far only recognized BH3-only protein in *C. elegans*, is required for all developmentally programmed cell deaths in this organism. In contrast, a number of BH3-only proteins have already been identified in mammals: Blk, Bad, Bik, Hrk, Bid, Bim, Noxa and Puma. Experiments with knock-out mice have shown that different apoptotic stimuli require distinct BH3-only proteins for their initiation. (Huang and Strasser, 2000, *supra*). For example, Bim is essential for apoptosis induced by cytokine withdrawal or antigen receptor stimulation, but is dispensable for cell death induced by glucocorticoids (Bouillet *et al., Science 286:* 1735, 1999; Bouillet et al., *Nature 415*, 922, 2002). In contrast, Bid is involved in Fas-induced killing of hepatocytes (Yin *et al., Nature 400:* 886, 1999). Moreover, different cell types may require distinct BH3-only proteins for their developmentally programmed death. Consistent with this idea, Bim-deficient mice have an abnormal accumulation of lymphoid and myeloid cells but erythropoiesis appears normal

(Bouillet *et al.*, 1999, *supra*). These results indicate that individual mammalian BH3-only proteins have specific functions.

The pro-apoptotic activity of BH3-only proteins is subject to stringent control. In *C. elegans*, EGL-1 is regulated by the transcriptional represser TRA-1A in a group of neurons that is required for egg-laying (Conradt and Horvitz, *Cell 93:* 519,1998). Some mammalian BH3-only proteins are also subject to transcriptional regulation. For example, Noxa was discovered as a p53-inducible gene and is therefore a prime candidate for mediating DNA damage-induced apoptosis (Oda *et al., Science 288:* 1053, 2000). Several mammalian BH3-only proteins can also be regulated post-translationally (Huang and Strasser, 2000, *supra*). In growth factor-stimulated cells, Bad is phosphorylated and sequestered away from pro-survival Bcl-2 family members by binding to 14-3-3 scaffold proteins (Zha *et al., Cell 87:* 619, 1996). In healthy cells, Bim is sequestered to the microtubular dynein motor complex by binding to dynein light chain, DLC1/LC8 (Puthalakath *et al., Mol. Cell 3:* 287, 1999). Certain apoptotic stimuli, such as UV-radiation or treatment with taxol, free Bim (still bound to DLC1) and allow it to translocate to, bind and inactivate pro-survival Bcl-2 family members. This process occurs independently of the cell death executioner cysteine proteases (caspases) and therefore constitutes an upstream signalling event in apoptosis (Puthalakath *et al.*, 1999, *supra*). In contrast, the pro-apoptotic activity of Bid is unleashed upon cleavage by a variety of caspases (e.g. caspase-8) or by the serine protease granzyme B (Li *et al., Cell 94:* 491-501, 1998; Luo *et al., Cell 94:* 481-490, 1998), indicating that it functions as part of an amplification mechanism rather than as an initiator of apoptosis. These observations demonstrate that through sequestration to specific sites in the cell, different BH3-only proteins function as sensors for distinct forms of intra-cellular stress.

In work leading to the present invention, the inventors sought novel BH3-only proteins which played a role in embryogenesis. In accordance with the present invention, the inventors cloned "Bmf" (Bcl-2 modifying factor) which was identified through yeast 2-hybrid screening of a day 17 mouse embryonic library using Mcl-1 as bait. Bmf is proposed to induce cell death and act as a "death-ligand" for certain or all members of the pro-survival Bcl-2 family. The identification of this new gene permits the identification

and rational design of a range of products for use in therapy, diagnosis, antibody generation and involving modulation of physiological cell death. These therapeutic molecules may act as either antagonists or agonists of Bmf's function and will be useful in cancer, autoimmune or degenerative disease therapy.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

Nucleotide and amino acid sequences are referred to by a sequence identifier number (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1 (SEQ ID NO:1), <400>2 (SEQ ID NO:2), etc. A sequence listing is provided after the claims.

Specific mutations in an amino acid sequence are represented herein as "X_1nX_2" where X_1 is the original amino acid residue before mutation, n is the residue number and X_2 is the mutant amino acid. Reference to Xn is a reference to a particular amino acid in an amino acid sequence where X is the amino acid and n is the residue number. The abbreviation X may be to the three letter or single letter amino acid code.

The present invention is predicated in part on the identification of a novel member of the pro-survival Bcl-2 family. This protein is referred to herein as "Bcl-2 modifying factor" or "Bmf". The protein was identified by yeast 2-hybrid screening of a mouse embryonic library using Mcl-1 as bait. Bmf is an apoptosis-inducing BH3-only protein and is activated by anoikis.

Accordingly, one aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having one or more of the identifying characteristics of Bmf or a derivative or homolog thereof.

Another aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID

NO:6 or SEQ ID NO:8 or a derivative or homolog thereof or having at least about 45% or greater similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or a derivative or homolog thereof.

Yet another aspect of the present invention contemplates a nucleic acid molecule comprising a nucleotide sequence substantially as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or a derivative or homolog thereof capable of hybridising to one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 under low stringency conditions and which encodes an amino acid sequence corresponding to an amino acid sequence set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or a sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or 8.

Still yet another aspect of the present invention contemplates a nucleic acid molecule comprising a sequence of nucleotides substantially as set forth in SEQ ID NOS:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7.

Still another aspect of the present invention is directed to an isolated nucleic acid molecule encoding *bmf* or a derivative thereof, said nucleic acid molecule selected from the list consisting of:-

(i) a nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or a derivative or homolog thereof or having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8;

(ii) a nucleic acid molecule comprising a nucleotide sequence substantially as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or a derivative or homolog thereof;

(iii) a nucleic acid molecule capable of hybridizing under low stringency conditions to the nucleotide sequence substantially as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 a derivative or homolog and encoding an amino acid sequence corresponding to an amino acid sequence as set forth in one of SEQ ID NO:SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 a derivative or homolog or a sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8;

(iv) a nucleic acid molecule capable of hybridizing to the nucleic acid molecule of paragraphs (i) or (ii) or (iii) under low stringency conditions and encoding an amino acid sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8; and

(v) a derivative or mammalian homolog of the nucleic acid molecule of paragraphs (i) or (ii) or (iii) or (iv).

A further aspect of the present invention is directed to an isolated polypeptide selected from the list consisting of:-

(i) a polypeptide having an amino acid sequence substantially as set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or derivative or homolog thereof or a sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8;

(ii) a polypeptide encoded by a nucleotide sequence substantially as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or derivative or homolog thereof or a sequence encoding an amino acid sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8;

(iii) a polypeptide encoded by a nucleic acid molecule capable of hybridizing to the nucleotide sequence as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or derivative or homolog thereof under low stringency conditions and which encodes an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or derivative or homolog thereof or an amino acid sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8;

(iv) a polypeptide as defined in paragraphs (i) or (ii) or (iii) in homodimeric form; and

(v) a polypeptide as defined in paragraphs (i) or (ii) or (iii) in heterodimeric form.

Yet another aspect of the present invention provides a method of producing a genetically modified non-human animal, said method comprising introducing into embryonic stem cells of an animal a genetic construct comprising a *bmf* nucleotide sequence carrying a single or multiple nucleotide substitution, addition and/or deletion or inversion or insertion wherein there is sufficient *bmf* nucleotide sequences to promote homologous recombination with a *bmf* gene within the genome of said embryonic stem cells selecting for said homologous recombination and selecting embryonic stem cells which carry a mutated *bmf* gene and then generating a genetically modified animal from said embryonic stem cell.

BRIEF DESCRIPTION OF THE FIGURE

Figure 1 is a representation showing Bmf, a novel mammalian BH3-only protein. **(A)** Predicted amino acid sequence of mouse and human Bmf. The nine amino acids that are conserved with the dynein light chain-binding motif of Bim are indicated by a box marked with a single asterisk (*). The short BH3 region, identified by hidden Markov modeling (Krogh *et al., J. Mol. Biol. 235:* 1501, 1994), is indicated by a box marked with two asterisks (**). **(B)** Alignment of the BH3 region of Bmf with other pro-apoptotic Bcl-2 family members. Black boxes indicate identical amino acids and grey boxes indicate similar residues. **(C)** Wild-type Bmf, but not a BH3 mutant, binds pro-survival Bcl-2 and Bcl-w. Co-immunoprecipitation experiments were carried out as previously described (Puthalakath *et al.*, 1999, *supra*). Briefly, 293T cells were transiently co-transfected with expression constructs for FLAG-tagged Bcl-2 (or Bcl-w) and EE(Glu-Glu)-tagged Bmf or L138A mutant Bmf. Cells were metabolically labeled with ^{35}S-methionine 24 hours after transfection and harvested after overnight culture. Volumes of cell lysates with equivalent trichloroacetic acid (TCA)-precipitable ^{35}S counts were used for immunoprecipitations with mAbs to the FLAG or EE epitope tags. **(D)** Interaction of endogenous Bmf with Bcl-2 in MCF-7 cells. Lysates from 10^7 MCF-7 cells, prepared in lysis buffer containing 1% v/v Triton X-100, were immunoprecipitated either with Bcl-2-100 (anti-human Bcl-2) mAb or an isotype matched control mAb coupled to sepharose. Bound proteins were eluted from the beads by boiling in Laemmli buffer (non reducing), size fractionated on SDS-PAGE and transferred onto nitrocellulose filters. Western blotting was performed with a rat anti-Bmf mAb (9G10). The asterisk (*) indicates the light chain of the mAb used for immunoprecipitation. **(E)** Wild-type Bmf, but not a BH3 mutant, kills L929 fibroblasts. L929 fibroblasts were transfected with empty vector, expression constructs for hygromycin resistance alone, or with wild-type Bmf, a BH3 mutant (L138A) of Bmf or Bmf lacking its BH3 domain. Transfected cells were plated in medium containing hygromycin and resulting drug-resistant colonies counted after 10-14 days. Values are means (+/-SD) of three independent experiments. **(F and G)** Expression of Bmf in cell lines and tissues. For Northern blot analysis (F), 4 μg of poly A^+ RNA from various cell lines or from mouse embryos (embryonic day 9 to 1-day after birth) were electrophoresed, blotted and probed

with a mouse *bmf* cDNA probe. Probing with a *gapdh* cDNA clone was used as the loading control. For Western blot analysis (G), 50 μg of total protein from various mouse tissues was size-fractionated by SDS-PAGE, electroblotted onto nitrocellulose filters and probed with affinity-purified rabbit polyclonal antibodies to Bmf. Probing with a monoclonal antibody to HSP70 served as the loading control.

Figure 2 is a representation showing Bmf is regulated by interaction with DLC2. **(A)** Expression of *bmf* mRNA in thymocytes treated with various apoptotic stimuli. Total RNA was isolated from thymocytes (freshly isolated) or at the indicated time points after culture in the absence of cytokines or treatment with dexamethasone (1 μM), γ-radiation (10 Gy) or ionomycin (1 μg/mL). These conditions all induce substantial apoptosis and, hence, no RNA could be harvested after 7 hours of treatment. Then 2 μg RNA was reverse transcribed using AMV reverse transcriptase. Five fold dilutions of the cDNA were subjected to PCR analysis using *bmf* specific primers. After transfer of the PCR products, nitrocellulose filters were probed with a ^{32}P-labeled internal *bmf* oligonucleotide probe. **(B)** Bmf binds to DLC2 through its dynein light chain binding region. Co-immunoprecipitation experiments were performed as described in the legend to Figure 1C, from lysates of 293T cells transiently expressing FLAG-tagged DLC2 and EE-tagged wt Bmf, a BH3 mutant (L138A) of Bmf or DLC binding region mutants of Bmf (A69P or AAA), Bid or Bax. The asterisk (*) indicates the light chain of the mAb used for immunoprecipitation. **(C)** Interaction with DLC2 regulates the pro-apoptotic potency of Bmf. FDC-P1 cells stably expressing Bcl-2 plus EE-tagged wt Bmf, a BH3 mutant (L138A) of Bmf or DLC binding region mutants of Bmf (A69P or AAA) were deprived of IL-3 for 1-6 days. Cell viability was assessed by propidium iodide staining and flow cytometric analysis. Values are means (+/-SD) of three independent experiments done with four independent clones of each genotype.

Figure 3 is a photographic representation showing that Bmf associates with the actin-based myosin V motor complex through DLC2. **(A)** Lysates from 10^7 MCF-7 cells were separated into P1, P2 and S fractions. Proteins from each fraction were then size-fractionated by SDS-PAGE, transferred onto nitrocellulose and probed with mAbs specific to Bmf, Bim_L (O'Reilly *et al.*, *Biotechniques 25:* 824, 1998), myosin V (Espreafico *et al.*, *J. Cell Biol.*

119: 1541, 1992) or dynein intermediate chain IC74 (Sigma). **(B)** MCF-7 cells were treated for 3 hours with either cytochalasin D (10 μM) or toxin B (10 ng/mL), then fractionated and processed as described under (A). **(C)** Characterization of novel mAbs that recognize both DLC1/LC8 and DLC2, or just DLC1/LC8. Extracts from 293T cells transiently expressing FLAG-tagged DLC1 or DLC2 were run on SDS-PAGE gels, electroblotted onto nitrocellulose membranes and probed with rat monoclonal antibodies 11F7 (which recognizes both DLC1 and DLC2) or 10D6 (which recognizes only DLC1). The faint bands of lower molecular weight marked by arrows indicate endogenous DLC1. **(D)** Myosin V is associated mostly with DLC2 whereas dynein predominantly associates with DLC1/LC8. Cytoplasmic dynein was enriched from MCF-7 cells (Paschal *et al., Methods Enzymol. 196:* 181, 1991) and myosin V was purified from mouse spleen (m) or chicken brain (c) (Cheney, *Methods Enzymol. 298:* 3, 1998). These enriched fractions were analyzed by Western blotting using rat mAbs 11F7 (recognizes DLC1/LC8 and DLC2) or 10D6 (recognizes only DLC1/LC8). Nitrocellulose membranes were probed with antibodies to myosin V or IC74 (Sigma) to demonstrate purity of the myosin and dynein motor fractions. **(E)** Extracts from mouse spleen cells (200 μg protein) were incubated for 3 hours at 4°C with recombinant GST or GST-tagged FADD, Bmf or Bim_L proteins, and the bound proteins recovered on glutathione sepharose beads. Bound proteins were eluted from the beads by boiling in Laemmli buffer (non-reducing), size-fractionated by SDS-PAGE and electro-blotted onto nitrocellulose membranes, which were probed with an antibody to myosin V (Espreafico *et al.*, 1992, *supra*). The nitrocellulose membrane was stained with amido black (bottom panel) to document that comparable amounts of proteins were used in the pull down experiments. **(F)** Lysates from 10^7 MCF-7 cells were fractionated through a 5-20% w/v sucrose gradient. The pellet and soluble fractions were analyzed by Western blotting for the presence of Bmf, Bim, DLC1/LC8 or DLC2.

Figure 4 is a photographic representation showing that Bmf and Bim are released from their sequestration sites in response to distinct apoptotic stimuli. **(A)** MCF-7 cells were cultured in the presence of the broad-spectrum caspase inhibitor zVAD-fmk (50 μM). Lysates from control (untreated) cells were compared with those from cells subjected to various apoptotic stimuli, including anoikis (culturing cells for 24 hours in suspension on

poly-hema coated bacterial Petri dishes), UV-irradiation (100 J/m^2), paclitaxel (taxol 1 μM). Lysates of 10^7 cells were fractionated through sucrose gradients. The pellet and soluble fractions were collected and analyzed by Western blotting for Bmf and Bim using specific monoclonal antibodies. **(B)** During anoikis, Bmf translocates to mitochondria and binds to Bcl-2. Mitochondria were purified as previously described from 2 x 10^8 healthy MCF-7 cells or cells subjected to anoikis. Mitochondrial proteins were extracted in lysis buffer containing 1% v/v Triton X-100 (Puthalakath *et al.*, 1999, *supra*). Immunoprecipitations were performed with anti-human Bcl-2 mAb (Bcl 2-100) bound to sepharose beads. Bound proteins were eluted by boiling the beads in Laemmli buffer (non-reducing), size-fractionated by SDS-PAGE, electroblotted onto nitrocellulose membranes and probed with mAbs to Bcl-2, Bmf or dynein light chains.

Figure 5A is a diagrammatic representation showing the genomic organization of the *bmf* gene locus of the mouse.

Figure 5B is a diagrammatic representation of a *bmf* targeting construct in NEB193neo or NEB193hygro for use in generating knock-out mice.

Single and three letter abbreviations used throughout the specification are defined below.

SINGLE AND THREE LETTER AMINO ACID ABBREVIATIONS

AMINO ACID	THREE-LETTER ABBREVIATION	ONE-LETTER SYMBOL
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V
Any residue	Xaa	X

A summary of sequence identifiers is provided below:-

SUMMARY OF SEQENCE IDENTIFIERS

SEQ ID NO:	DESCRIPTION
1	Nucleotide sequence of mouse *bmf*
2	Amino acid sequence of mouse Bmf
3	Nucleotide sequence of human *bmf*
4	Amino acid sequence of human Bmf
5	Nucleotide sequence of BH3 domain of mouse *bmf*
6	Amino acid sequence of BH3 domain of mouse Bmf
7	Nucleotide sequence of BH3 domain of human *bmf*
8	Amino acid sequence of BH3 domain of human *bmf*
9	Nucleotide sequence of mouse *bmf* promoter
10	Nucleotide sequence of human *bmf* promoter
11	5' sense primer
12	3' antisense primer
13	internal *bmf* primer
14	5' sense primer
15	3' antisense primer
16	internal primer
17	predicted amino acid sequence of mouse Bmf
18	predicted amino acid sequence of human Bmf
19	partial amino acid sequence of Bmf
20	partial amino acid sequence of Bim
21	partial amino acid sequence of EGL-1
22	partial amino acid sequence of Bak
23	partial amino acid sequence of Bax
24	partial amino acid sequence of Bid
25	partial amino acid sequence of Bik
26	partial amino acid sequence of Hrk
27	partial amino acid sequence of Bad

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated in part on the identification of a novel member of the Bcl-2 family of proteins. The protein is called "Bmf" for "Bcl-2 modifying factor". It is proposed that in healthy cells, Bmf is sequestered to the actin-based myosin V motor complex by binding to a dynein light chain and in particular dynein light chain 2 (DLC2). It is further proposed that certain apoptotic stimuli, such as anoikis, release Bmf from the myosin V motor complex allowing it to translocate and bind to Bcl-2. Consequently, Bmf functions as a sensor of intracellular damage by sequestration to motor complexes on distinct cytoskeletal strcutures.

Accordingly, one aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or a derivative or homolog thereof or having at least about 45% or greater similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or a derivative or homolog thereof.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, nucleotide and sequence comparisons are made at the level of identity rather than similarity.

Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence similarity", "sequence identity", "percentage of sequence similarity", "percentage of sequence

identity", "substantially similar" and "substantial identity". A "reference sequence" is at least 12 but frequently 15 to 18 and often at least 25 or above, such as 30 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (i.e. only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of typically 12 contiguous residues that is compared to a reference sequence. The comparison window may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerised implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e. resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as, for example, disclosed by Altschul *et al.* (*Nucl. Acids. Res. 25:* 3389, 1997). A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel *et al.* ("Current Protocols in Molecular Biology", John Wiley & Sons Inc., 1994-1998, Chapter 15).

The terms "sequence similarity" and "sequence identity" as used herein refers to the extent that sequences are identical or functionally or structurally similar on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity", for example, is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g. A, T, C, G, I) or the identical amino acid residue (e.g. Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched

positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For the purposes of the present invention, "sequence identity" will be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the reference manual accompanying the software. Similar comments apply in relation to sequence similarity.

Another aspect of the present invention contemplates a nucleic acid molecule comprising a nucleotide sequence substantially as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or a derivative or homolog thereof capable of hybridizing to one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 under low stringency conditions and which encodes an amino acid sequence corresponding to an amino acid sequence set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or a sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8.

More particularly, the present invention contemplates a nucleic acid molecule comprising a sequence of nucleotides substantially as set forth in SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7.

Preferably, the subject nucleic acid molecules encode a polypeptide having the identifying characteristics of Bmf or its homologs or derivatives including functional derivatives.

Reference herein to a low stringency includes and encompasses from at least about 0 to at least about 15% v/v formamide and from at least about 1 M to at least about 2 M salt for hybridization, and at least about 1 M to at least about 2 M salt for washing conditions. Generally, low stringency is at from about 25-30°C to about 42°C. The temperature may be altered and higher temperatures used to replace formamide and/or to give alternative stringency conditions. Alternative stringency conditions may be applied where necessary,

such as medium stringency, which includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5 M to at least about 0.9 M salt for hybridization, and at least about 0.5 M to at least about 0.9 M salt for washing conditions, or high stringency, which includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01 M to at least about 0.15 M salt for hybridization, and at least about 0.01 M to at least about 0.15 M salt for washing conditions. In general, washing is carried out $T_m = 69.3 + 0.41\ (G+C)\%$ (Marmur and Doty, *J. Mol. Biol. 5:* 109, 1962). However, the T_m of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (Bonner and Lasky, *Eur. J. Biochem. 46:* 83, 1974). Formamide is optional in these hybridization conditions. Accordingly, particularly preferred levels of stringency are defined as follows: low stringency is 6 x SSC buffer, 0.1% w/v SDS at 25-42°C; a moderate stringency is 2 x SSC buffer, 0.1% w/v SDS at a temperature in the range 20°C to 65°C; high stringency is 0.1 x SSC buffer, 0.1% w/v SDS at a temperature of at least 65°C.

The nucleic acid molecule according to this aspect of the present invention corresponds herein to "*bmf*". This gene has been determined in accordance with the present invention to induce apoptosis. The product of the *bmf* gene is referred to herein as "Bmf" without limiting this invention in any way, human *bmf* has been mapped to human chromosome location 15q14. Bmf is known as a "BH3-only" protein since the only Bcl-2 homology region which it contains is BH3. It thereby forms a novel member of a Bcl-2 related BH3-only pro-apoptotic group which also comprises, for example, Bik/Nbk, Bid, Bim and Hrk.

The nucleic acid molecule encoding *bmf* is preferably a sequence of deoxyribonucleic acids such as cDNA sequence, an mRNA sequence or a genomic sequence. A genomic sequence may also comprise exons and introns. A genomic sequence may also include a promoter region or other regulatory region. The *bmf* genetic sequence includes splice variants.

Reference hereinafter to "Bmf" and "*bmf*" should be understood as a reference to all forms of Bmf and *bmf*, respectively, including, by way of example, polypeptide and cDNA

isoforms of *bmf* which may be identified as arising from alternative splicing of *bmf* mRNA. Reference hereinafter to Bmf and *bmf* in the absence of a reference to its derivatives should be understood to include reference to its derivatives thereof including any splice variants.

The protein and/or gene is preferably from a human, primate, livestock animal (e.g. sheep, pig, cow, horse, donkey) laboratory test animal (e.g. mouse, rat, rabbit, guinea pig) companion animal (e.g. dog, cat), captive wild animal (e.g. fox, kangaroo, koala, deer), aves (e.g. chicken, geese, duck, emu, ostrich), reptile or fish.

Derivatives include fragments (such as peptides), parts, portions, chemical equivalents, mutants, homologs or mimetics from natural, synthetic or recombinant sources including fusion proteins. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences including fusions with other peptides, polypeptides or proteins. Mutants should be understood to include, but is not limited to, the specific Bmf or *bmf* mutant molecules described herein. Derivatives include, for example, peptides derived from the BH3 region, from the dynein binding region or from a site of phosphorylation. Peptides include, for example, molecules comprising at least 4 contiguous amino acids corresponding to at least 4 contiguous amino acids of Bmf as herein defined. Use of the term "polypeptides" herein should be understood to encompass peptides, polypeptides and proteins.

The derivatives of Bmf include fragments having particular epitopes or parts of the entire Bmf protein fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules. For example, Bmf or derivative thereof may be fused to a molecule to facilitate its entry into a cell. Analogues of Bmf contemplated herein include, but are not limited to,

modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecules or their analogues. Derivatives of nucleic acid sequences may similarly be derived from single or multiple nucleotide substitutions, deletions and/or additions including fusion with other nucleic acid molecules. The derivatives of the nucleic acid molecules of the present invention include oligonucleotides, PCR primers, antisense molecules, molecules suitable for use in co-suppression and fusion of nucleic acid molecules.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid, contemplated herein is shown in Table 1.

TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-	Cpro	L-N-methylasparagine	Nmasn
carboxylate		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-	Norb	L-N-methylglutamine	Nmgln
carboxylate		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-Nmethylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle
D-tryptophan	Dtrp	L-norvaline	Nva

D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
D-α-methylleucine	Dmleu	α-napthylalanine	Anap
D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis

D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-α-methylalanine	Mala
L-α-methylarginine	Marg	L-α-methylasparagine	Masn
L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr
L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe
N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe

1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc

Crosslinkers can be used, for example, to stabilize 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N_α-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

The nucleic acid molecule of the present invention is preferably in isolated form or ligated to a vector, such as an expression vector. By "isolated" is meant a nucleic acid molecule having undergone at least one purification step and this is conveniently defined, for example, by a composition comprising at least about 10% subject nucleic acid molecule, preferably at least about 20%, more preferably at least about 30%, still more preferably at least about 40-50%, even still more preferably at least about 60-70%, yet even still more preferably 80-90% or greater of subject nucleic acid molecule relative to other components as determined by molecular weight, encoding activity, nucleotide sequence, base composition or other convenient means. The nucleic acid molecule of the present invention may also be considered, in a preferred embodiment, to be biologically pure.

In a particularly preferred embodiment, the nucleotide sequence corresponding to *bmf* is a cDNA sequence comprising a sequence of nucleotides as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or is a derivative or homolog thereof including a nucleotide sequence having similarity to one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 and which encodes an amino acid sequence

corresponding to an amino acid sequence as set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or a sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8.

A derivative of the nucleic acid molecule of the present invention also includes nucleic acid molecules capable of hybridizing to the nucleotide sequences as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 under low stringency conditions. Preferably, said low stringency is at 42°C.

In another embodiment, the present invention is directed to an isolated nucleic acid molecule encoding *bmf* or a derivative thereof, said nucleic acid molecule selected from the list consisting of:-

(i) a nucleic acid molecule comprising a nucleotide sequence encoding the amino acid sequence set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or a derivative or homolog thereof or having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8;

(ii) a nucleic acid molecule comprising a nucleotide sequence substantially as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or a derivative or homolog thereof;

(iii) a nucleic acid molecule capable of hybridizing under low stringency conditions to the nucleotide sequence substantially as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 a derivative or homolog and encoding an amino acid sequence corresponding to an amino acid sequence as set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or a derivative or homolog or a sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8;

(iv) a nucleic acid molecule capable of hybridizing to the nucleic acid molecule of paragraphs (i) or (iii) under low stringency conditions and encoding an amino acid sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8; and

(v) a derivative or mammalian homolog of the nucleic acid molecule of paragraphs (i) or (ii) or (iii) or (iv).

Reference here to an ability to hybridize to a particular sequence (e.g. SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7) also includes, in the alternative, an ability to hybridize to its complementary form. In other words, nucleic acid molecules are encompassed which hybridize to SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or their complementary forms.

The nucleic acid molecule may be ligated to an expression vector capable of expression in a prokaryotic cell (e.g. *E.coli*) or a eukaryotic cell (e.g. yeast cells, fungal cells, insect cells, mammalian cells or plant cells). The nucleic acid molecule may be ligated or fused or otherwise associated with a nucleic acid molecule encoding another entity such as, for example, a signal peptide, a cytokine or other member of the Bcl-2 family.

The present invention extends to the promoter for *bmf* from murine or other mammalian species. Nucleotide sequences comprising the murine and human *bmf* promoters are shown in SEQ ID NO:9 and SEQ ID NO:10, respectively. The present invention extends to mutants and derivatives of these promoters and their use in genetic constructs, gene therapy and in generating genetically modified animals. A mutant or derivative of a promoter includes one which comprises a nucleotide sequence having at least 70% similarity to SEQ ID NOS:9 or 10 or which is capable of hybridizing to SEQ ID NO:9 or SEQ ID NO:10 or their complementary forms under low stringency conditions.

The present invention extends to the expression product of the nucleic acid molecule hereinbefore defined.

The expression product is Bmf having an amino acid sequence set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or is a derivative or homolog thereof as defined above or is a mammalian homolog having an amino acid sequence of at least about 45% similarity to the amino acid sequence set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or derivative or homolog thereof.

Another aspect of the present invention is directed to an isolated polypeptide selected from the list consisting of:-

(i) a polypeptide having an amino acid sequence substantially as set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or derivative or homolog thereof or a sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8;

(ii) a polypeptide encoded by a nucleotide sequence substantially as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or derivative or homolog thereof or a sequence encoding an amino acid sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8;

(iii) a polypeptide encoded by a nucleic acid molecule capable of hybridizing to the nucleotide sequence as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or derivative or homolog thereof under low stringency conditions and which encodes an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or derivative or homolog thereof or an amino acid sequence having at least about 45% similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8;

(iv) a polypeptide as defined in paragraphs (i) or (ii) or (iii) in homodimeric form; and

(v) a polypeptide as defined in paragraphs (i) or (ii) or (iii) in heterodimeric form.

As defined earlier, the present invention extends to peptides or derivatives thereof of Bmf. Preferably, said peptide comprises at least 5 contiguous amino acids of the polypeptide defined in SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8. The present invention also extends to nucleic acid molecules encoding the peptides of the present invention.

Another aspect of the present invention provides a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide having one or more of the identifying characteristics of Bmf or a derivative or homolog thereof.

Reference herein to "identifying characteristics" of Bmf includes one or more of the following features:-

(i) a polypeptide which induces apoptosis;

(ii) a polypeptide having an amino acid sequence substantially as set forth in SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or a derivative or homolog thereof;

(iii) a polypeptide having an amino acid sequence of at least 45% similarity to SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8;

(iv) a polypeptide as defined in paragraph (ii) or (iii) which induces apoptosis;

(v) a polypeptide encoded by a nucleic acid sequence substantially as set forth in SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or derivative or homolog thereof;

(vi) a polypeptide encoded by a nucleic acid molecule capable of hybridizing to the nucleotide sequence as set forth in one of SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 under low stringency conditions;

(vii) a polypeptide as defined in paragraph (v) or (vi) which induces apoptosis; and

(viii) a non-apoptosis inducing derivative of the polypeptide defined in paragraphs (i) to (vii).

The present invention should be understood to extend to the expression product of the nucleic acid molecule according to this aspect of the present invention.

Although not intending to limit the invention to any one theory or mode of action, the BH3 region is responsible for some of the cytotoxic actions of Bmf. The BH3 region forms an amphipathic α-helix that interacts with the elongated hydrophobic cleft formed by the BH1, BH2 and BH3 regions of pro-survival molecules such as, for example, Bcl-x_L. The pro-apoptotic action of Bmf reflects its ability to bind to the anti-apoptotic members of the Bcl-2 family.

Still without limiting the invention to any one theory or mode of action, the pro-apoptotic activity of Bmf is thought to be regulated both at the transcriptional level and at the post-translational level. Sequence analysis of the non-coding 5' region of *Bmf* has revealed a number of putative binding sites for transcription factors such as AP1. Bmf is proposed to interact *via* a dynein light chain such as DLC2. A dynein light chain is a highly conserved protein which is a component of the myosin V motor complex.

The interaction of Bmf with the myosin V motor complex regulates the pro-apoptotic activity of Bmf. Single or multiple amino acid mutations in Bmf which abolish binding to the dynein light chain are encompassed by the present invention.

Accordingly, a related aspect of the present invention is directed to a variant of an isolated *bmf* nucleic acid molecule comprising one or more nucleotide mutations in said nucleic acid molecule resulting in at least one amino acid addition, substitution and/or deletion to the polypeptide encoded by said variant wherein said polypeptide cannot bind, couple or otherwise associate with a dynein light chain, such as DLC2.

Preferably, the mutation results in an altered amino acid sequence in the region which binds the dynein light chain. The present invention should be understood to extend to variants of Bmf comprising a mutation resulting in an amino acid addition, substitution and/or deletion in a region functionally equivalent to the regions hereinbefore defined.

Accordingly, the present invention is more particularly directed to a variant of an isolated *bmf* nucleic acid molecule comprising one or more nucleotide mutations in said nucleic acid molecule resulting in at least one amino acid addition, substitution and/or deletion in the region of the polypeptide encoded by said variant which binds the dynein light chain wherein said polypeptide cannot bind, couple or otherwise associate with a dynein light chain.

Mutations contemplated by the present invention which occur in combination with one or more mutations in another location are also contemplated by the present invention.

The present invention extends to the expression products of the nucleic acid molecule variants defined according to this aspect of the present invention.

Accordingly, the present invention is directed to a variant of an isolated Bmf polypeptide comprising at least one amino acid addition, substitution and/or deletion wherein said polypeptide cannot bind, couple or otherwise associate with the dynein light chain.

The present invention extends to derivatives of the nucleic acid molecules and polypeptides according to this aspect of the present invention. The term "derivatives" should be understood as previously defined.

As hereinbefore defined, reference to "Bmf" and "*bmf*" should be understood to include reference to the variant molecules defined according to this aspect of the present invention.

The Bmf of the present invention may be in multimeric form meaning that two or more molecules are associated together. Where the same Bmf molecules are associated together, the complex is a homomultimer. An example of a homomultimer is a homodimer. Where at least one Bmf is associated with at least one non-Bmf molecule, then the complex is a heteromultimer such as a heterodimer. A heteromultimer may include a molecule of another member of the Bcl-2 family or other molecule capable of modulating apoptosis. Furthermore, the present invention contemplates fusion, or hybrids or heteromeric dimers between Bmf and other molecules such as Bim.

The present invention contemplates, therefore, a method for modulating expression of *bmf* in a mammal, said method comprising administering to said mammal a modulating effective amount of an agent for a time and under conditions sufficient to up-regulate or down-regulate or otherwise modulate expression of *bmf*. For example, *bmf* antisense sequences such as oligonucleotides may be introduced into a cell to enhance the ability of that cell to survive. Conversely, a nucleic acid molecule encoding Bmf or a derivative thereof may be introduced to decrease the survival capacity of any cell expressing the endogenous *bmf* gene. Modulation of the expression of *bmf* should be understood to extend to modulating transcriptional and translation events such as the splicing pattern of *Bmf* RNA.

Another aspect of the present invention contemplates a method of modulating activity of Bmf in a mammal, said method comprising administering to said mammal a modulating effective amount of an agent for a time and under conditions sufficient to increase or decrease Bmf activity.

Modulation of said activity by the administration of an agent to a mammal can be achieved by one of several techniques, including but in no way limited to introducing into said mammal a proteinaceous or non-proteinaceous molecule which:

(i) modulates expression of *bmf*;

(ii) functions as an antagonist of Bmf; and

(iii) functions as an agonist of Bim.

Said proteinaceous molecule may be derived from natural or recombinant sources including fusion proteins or following, for example, natural product screening. Said non-proteinaceous molecule may be, for example, a nucleic acid molecule or may be derived from natural sources, such as for example natural product screening or may be chemically synthesized. The present invention contemplates chemical analogues of Bmf capable of acting as agonists or antagonists of Bmf. Chemical agonists may not necessarily be derived from Bmf but may share certain conformational similarities. Alternatively, chemical agonists may be specifically designed to mimic certain physiochemical properties of Bmf. Antagonists may be any compound capable of blocking, inhibiting or otherwise preventing Bmf from carrying out its normal or pathological biological functions. Antagonists include, but are not limited to parts of Bmf or peptides thereof, monoclonal antibodies specific for Bmf or parts of Bmf, and antisense nucleic acids or oligonucleotides which prevent transcription or translation of *bmf* genes or mRNA in mammalian cells. Agonists of Bmf and *bmf* include, for example, the derivative or variant molecules or peptides hereinbefore defined which interact with anti-apoptotic molecules such as Bcl-2, to prevent their functional activity thereby promoting apoptosis. Agonists may also include molecules capable of disrupting or preventing binding of Bmf to the dynein light chain or the interaction of dynein light chain with dynein intermediate chain.

Said proteinaceous or non-proteinaceous molecule may act either directly or indirectly to modulate the expression of *bmf* or the activity of Bmf. Said molecule acts directly if it associates with *Bmf* or Bmf to modulate the expression or activity of *bmf* or Bmf. Said molecule acts indirectly if it associates with a molecule other than *bmf* or Bmf which other molecule either directly or indirectly modulates the expression or activity of *bmf* or Bmf.

Accordingly, the method of the present invention encompasses the regulation of *bmf* or Bmf expression or activity *via* the induction of a cascade of regulatory steps which lead to the regulation of *bmf* or Bmf expression or activity.

Increased *bmf* expression or Bmf activity is useful, for example, for treatment or prophylaxis in conditions such as cancer and deletion of autoreactive lymphocytes in autoimmune disease. Decreased *bmf* expression or Bmf activity is useful in regulating inhibition or prevention of cell death or degeneration such as under cytotoxic conditions during, for example, γ-irradiation and chemotherapy or during HIV/AIDS or other viral infections, ischaemia or myocardial infarction. Since Bmf is expressed in germ cells, modulating *bmf* expression or Bmf activity is useful, for example, as a contraceptive or method of sterilisation by preventing generation of fertile sperm.

Another aspect of the present invention contemplates a method of modulating apoptosis in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a nucleotide sequence encoding *bmf*.

Yet another aspect of the present invention contemplates a method of modulating apoptosis in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of Bmf.

Still another aspect of the present invention contemplates a method of modulating apoptosis in a mammal, said method comprising administering to said mammal an effective amount of Bmf or *bmf* or derivative thereof.

The Bmf, *bmf* or derivative thereof or agent used may also be linked to a targeting means such as a monoclonal antibody, which provides specific delivery of the Bmf, *bmf* or agent to the target cells.

In a preferred embodiment of the present invention, the Bmf, *bmf* or agent used in the method is linked to an antibody specific for said target cells to enable specific delivery to these cells.

Modulation of Bmf or *bmf* may be accompanied simultaneously or sequentially with the modulation of other molecules or genes such as but not limited to *bim* or Bim.

Administration of the Bmf, *bmf* or agent, in the form of a pharmaceutical composition, may be performed by any convenient means. Bmf, *bmf* or agent of the pharmaceutical composition are contemplated to exhibit therapeutic activity when administered in an amount which depends on the particular case. The variation depends, for example, on the human or animal and the Bmf, *bmf* or agent chosen. A broad range of doses may be applicable. Considering a patient, for example, from about 0.01 mg to about 10 mg of Bmf or agent may be administered per kilogram of body weight per day. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, weekly, monthly or other suitable time intervals or the dose may be proportionally reduced as indicated by the exigencies of the situation. The Bmf or agent may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intranasal, intraperitoneal, intramuscular, subcutaneous, intradermal or suppository routes or implanting (e.g. using slow release molecules). With particular reference to use of Bmf or agent, these peptides may be administered in the form of pharmaceutically acceptable nontoxic salts, such as acid addition salts or metal complexes, e.g. with zinc, iron or the like (which are considered as salts for purposes of this application). Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succinate, malate, ascorbate, tartrate and the like. If the active ingredient is to be administered in tablet form, the tablet may contain a binder such as tragacanth, corn starch or gelatin; a disintegrating agent, such as alginic acid; and a lubricant, such as magnesium stearate.

A further aspect of the present invention relates to the use of the invention in relation to mammalian disease conditions. For example, the present invention is particularly

applicable to, but in no way limited to, use in therapy or prophylaxis in relation to cancer, degenerative diseases, autoimmune disorders, viral infections or for germ cell regulation.

Accordingly, another aspect of the present invention relates to a method of treating a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of *bmf* wherein said modulation results in modulation of apoptosis.

In another aspect, the present invention relates to a method of treating a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of Bmf wherein said modulation results in modulation of apoptosis.

In another aspect, the present invention relates to a method of treating a mammal, said method comprising administering to said mammal an effective amount of Bmf or derivative thereof for a time and under conditions sufficient to modulate apoptosis.

Yet another aspect of the present invention relates to a method of treating a mammal, said method comprising administering to said mammal an effective amount of *bmf* or derivative thereof for a time and under conditions sufficient to modulate apoptosis.

In yet another aspect, the present invention relates to the use of an agent capable of modulating the expression of *bmf* or derivative thereof in the manufacture of a medicament for the modulation of apoptosis.

Another aspect of the present invention relates to the use of an agent capable of modulating the expression of Bmf or derivative thereof in the manufacture of a medicament for the modulation of apoptosis.

A further aspect of the present invention relates to the use of Bmf or *bmf* or derivative thereof in the manufacture of a medicament for the modulation of apoptosis.

Still yet another aspect of the present invention relates to agents for use in modulating *bmf* expression wherein modulating expression of said *bmf* modulates apoptosis.

A further aspect of the present invention relates to agents for use in modulating Bmf expression wherein modulating expression of said Bmf modulates apoptosis.

Another aspect of the present invention relates to Bmf or *bmf* or derivative thereof for use in modulating apoptosis.

In a related aspect of the present invention, the mammal undergoing treatment may be human or an animal in need of therapeutic of prophylactic treatment.

In yet another further aspect, the present invention contemplates a pharmaceutical composition comprising *bmf*, Bmf or derivative thereof or an agent capable of modulating *bmf* expression or Bmf activity together with one or more pharmaceutically acceptable carriers and/or diluents. *bmf*, Bmf or said agent are referred to as the active ingredients.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions (where water soluble) and sterile powders for the extemporaneous preparation of sterile injectable solutions. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dilution medium comprising, for example, water, ethanol, polyol (for example, glycerol, propylene glycol and liquid polyethylene glycol, and the like), suitable mixtures thereof and vegetable oils. The proper fluidity can be maintained, for example, by the use of surfactants. The preventions of the action of microorganisms can be brought about by various anti-bacterial and anti-fungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thirmerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the

use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with the active ingredient and optionally other active ingredients as required, followed by filtered sterilization or other appropriate means of sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, suitable methods of preparation include vacuum drying and the freeze-drying technique which yield a powder of active ingredient plus any additionally desired ingredient.

When *bmf*, Bmf and/or Bmf modulators are suitably protected, they may be orally administered, for example, with an inert diluent or with an assimilable edible carrier, or it may be enclosed in hard or soft shell gelatin capsule, or it may be compressed into tablets, or it may be incorporated directly with the food of the diet or administered *via* breast milk. For oral therapeutic administration, the active ingredient may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers and the like. Such compositions and preparations should contain at least 1% by weight of active compound. The percentage of the compositions and preparations may, of course, be varied and may conveniently be between about 5 to about 80% of the weight of the unit. The amount of active compound in such therapeutically useful compositions is such that a suitable dosage will be obtained. Preferred compositions or preparations according to the present invention are prepared so that an oral dosage unit form contains between about 0.1 μg and 200 mg of active compound. Alternative dosage amounts include from about 1 μg to about 1000 mg and from about 10 μg to about 500 mg. These dosages may be per individual or per kg body weight. Administration may be per hour, day, week, month or year.

The tablets, troches, pills, capsules, creams and the like may also contain the components as listed hereafter. A binder such as gum, acacia, corn starch or gelatin; excipients such as dicalcium phosphate; a disintegrating agent such as corn starch, potato starch, alginic acid

and the like; a lubricant such as magnesium stearate; and a sweetening agent such as sucrose, lactose or saccharin may be added or a flavouring agent such as peppermint, oil of wintergreen or cherry flavouring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills or capsules may be coated with shellac, sugar or both. A syrup or elixir may contain the active compound, sucrose as a sweetening agent, methyl and propylparabens as preservatives, a dye and flavouring such as cherry or orange flavour. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compound(s) may be incorporated into sustained-release preparations and formulations.

Pharmaceutically acceptable carriers and/or diluents include any and all solvents, dispersion media, coatings, anti-bacterial and anti-fungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art and except insofar as any conventional media or agent is incompatible with the active ingredient, their use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the novel dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active material for the treatment of disease in living subjects having a diseased condition in which bodily health is impaired as herein disclosed in detail.

The principal active ingredient is compounded for convenient and effective administration in effective amounts with a suitable pharmaceutically acceptable carrier in dosage unit form as hereinbefore disclosed. A unit dosage form can, for example, contain the principal active compound in amounts ranging from 0.5 μg to about 2000 mg. Expressed in proportions, the active compound is generally present in from about 0.5 μg to about 2000 mg/ml of carrier. In the case of compositions containing supplementary active ingredients, the dosages are determined by reference to the usual dose and manner of administration of the said ingredients.

The pharmaceutical composition may also comprise genetic molecules such as a vector capable of transfecting target cells where the vector carries a nucleic acid molecule capable of modulating *bmf* expression or Bmf activity. The vector may, for example, be a viral vector.

Conditions requiring modulation of physiological cell death include enhancing survival of cells utilising, for example, antisense sequence in patients with neurodegenerative diseases, myocardial infarction, muscular degenerative disease, hypoxia, ischaemia, HIV infection or for prolonging the survival of cells being transplanted for treatment of disease. Alternatively, the molecules of the present invention are useful for, for example, reducing the survival capacity of tumour cells or autoreactive lymphocytes. The antisense sequence may also be used for modifying *in vitro* behaviour of cells, for example, as part of a protocol to develop novel lines from cell types having unidentified growth factor requirements; for facilitating isolation of hybridoma cells producing monoclonal antibodies, as described below; and for enhancing survival of cells from primary explants while they are being genetically modified.

Still another aspect of the present invention is directed to an immunointeractive molecule comprising an antigen binding portion having specificity for Bmf or *bmf* or derivative thereof.

Reference to "immunointeractive molecule" should be understood as a reference to any molecule comprising an antigen binding portion or a derivative of said molecule. Examples of molecules contemplated by this aspect of the present invention include, but are not limited to, monoclonal and polyclonal antibodies (including synthetic antibodies, hybrid antibodies, humanized antibodies, catalytic antibodies) and T cell antigen receptor binding molecules. Preferably, said immunoreactive molecule is a monoclonal antibody.

According to this preferred embodiment, there is provided a monoclonal antibody having specificity for Bmf or *bmf* or derivative thereof.

Reference to a molecule "having specificity for Bmf or *bmf*" should be understood as a reference to a molecule, such as a monoclonal antibody, having specificity for any one or more epitopes of Bmf or *bmf*. These epitopes may be conformational epitopes, linear epitopes or a combination of conformational and linear epitopes of either the native Bmf or *bmf* molecule or the denatured molecule.

More preferably, there is provided a monoclonal antibody having specificity for Bmf.

The immunointeractive molecules of the present invention may be naturally occurring, synthetic or recombinantly produced. For example, monoclonal or polyclonal antibodies may be selected from naturally occurring antibodies to Bmf or *bmf* or may be specifically raised to Bmf or *bmf*. In the case of the latter, Bmf or *bmf* may first need to be associated with a carrier molecule. The antibodies and/or recombinant Bmf of the present invention are particularly useful as therapeutic or diagnostic agents. Alternatively, fragments of antibodies may be used such as Fab fragments. Furthermore, the present invention extends to recombinant and synthetic antibodies, to antibody hybrids and to antibodies raised against non-Bmf antigens but which are cross-reactive with any one or more Bmf epitopes. A "synthetic antibody" is considered herein to include fragments and hybrids of antibodies. The antibodies of this aspect of the present invention are particularly useful for immunotherapy and may also be used as a diagnostic tool for assessing apoptosis or monitoring the program of a therapeutic regime.

For example, Bmf and *bmf* can be used to screen for naturally occurring antibodies to Bmf and *bmf*, respectively. These may occur, for example in some degenerative disorders.

For example, specific antibodies can be used to screen for Bmf proteins. The latter would be important, for example, as a means for screening for levels of Bmf in a cell extract or other biological fluid or purifying Bmf made by recombinant means from culture supernatant fluid. Techniques for the assays contemplated herein are known in the art and include, for example, sandwich assays, ELISA and flow cytometry.

It is within the scope of this invention to include any second antibodies (monoclonal, polyclonal or fragments of antibodies) directed to the first mentioned antibodies discussed above. Both the first and second antibodies may be used in detection assays or a first antibody may be used with a commercially available anti-immunoglobulin antibody. An antibody as contemplated herein includes any antibody specific to any region of Bmf.

Both polyclonal and monoclonal antibodies are obtainable by immunization with the protein or peptide derivatives and either type is utilizable for immunoassays. The methods of obtaining both types of sera are well known in the art. Polyclonal sera are less preferred but are relatively easily prepared by injection of a suitable laboratory animal with an effective amount of Bmf, or antigenic parts thereof, collecting serum from the animal, and isolating specific sera by any of the known immunoadsorbent techniques. Although antibodies produced by this method are utilizable in virtually any type of immunoassay, they are generally less favoured because of the potential heterogeneity of the product.

The use of monoclonal antibodies in an immunoassay is particularly preferred because of the ability to produce them in large quantities and the homogeneity of the product. The preparation of hybridoma cell lines for monoclonal antibody production derived by fusing an immortal cell line and lymphocytes sensitized against the immunogenic preparation can be done by techniques which are well known to those who are skilled in the art. (See, for example, Douillard and Hoffman, Basic Facts about Hybridomas, in *Compendium of*

Immunology Vol. II, ed. by Schwartz, 1981; Kohler and Milstein, *Nature 256:* 495-499, 1975; Kohler and Milstein, *European Journal of Immunology 6:* 511-519, 1976).

Screening for immunointeractive molecules, such as antibodies, can be a time consuming and labour intensive process. However, the inventors have developed a rapid and efficient flow cytometric screening procedure for the identification of immunointereactive molecules, and in particular antibodies, directed to low abundance cytoplasmic proteins such as, but not limited to, Bmf.

The method according to this aspect of the present invention is based on the analysis of a population of cells, following the incubation of these cells with the antibody of interest together with or separately to a reporter molecule, said population of cells comprising both cells expressing the protein of interest and cells which do not express the protein of interest. This analysis is preferably flow cytometric analysis and the cells expressing the protein of interest are preferably transfected with a nucleic acid molecule encoding the protein of interest to thereby express high levels of said protein. Where the protein is a cytoplasmic protein the cells are permeabalized prior to incubation with the antibody of interest. By screening a population of cells comprising both cells which do not express and cells which do express the protein of interest, determination of which antibodies bind to the protein of interest is simplified since where the subject antibody is directed to the protein of interest, a double fluorescence peak is observed. The lower intensity peak represents background staining while the higher fluorescence intensity peak is the result of specific staining. Where the antibody being screened according to this method is not directed to the protein of interest, a single peak of low fluorescence intensity is observed. Antibodies not specific to the protein of interest but bound to some unknown epitope present in both populations of cells produces a single peak with high fluorscence intensity. This technique provides a rapid and accurate method of screening for immunointeractive molecules directed to low abundance intracytoplasmic molecules (O'Reilly, 1998, *supra*).

Accordingly, another aspect of the present invention provides a method of detecting an immunointeractive molecule, in a sample, specific for a protein of interest produced by a

cell, said method comprising contacting the sample to be tested with a population of cells comprising a defined ratio of cells producing the protein of interest and cells not producing the protein of interest for a time and under conditions sufficient for immunointeractive molecules, if present in said sample, to interact with said protein of interest and the subjecting said immunointeractive molecule-protein complex to detecting means.

Preferably said immunointeractive molecule is an antibody.

More preferably, said detecting means comprises an anti-immunoglobulin antibody labelled with a reporter molecule capable of giving a detectable signal. Even more preferably said reporter molecule is fluorochrome.

Reference to "sample" should be understood as a reference to any sample potentially comprising an immunointeractive molecule, such as an antibody. Said immunointeractive molecule may be produced by natural, recombinant or synthetic means.

The method of the present invention is predicated on subjecting the cells incubated with the sample of the present invention to flow cytometric analysis to produce a fluorescent signal wherein a differential fluorescent signal is indicative of antibody binding to the target protein expressed by said cells.

The method exemplified herein is directed, but not limited to, screening for immunointeractive molecules comprising an antigen binding site directed to epitopes of Bmf. The promyelomoncytic cell line FDC-P1 is transfected with a Bcl-2 expression construct and an EE (Glu-Glu) epitope-tagged Bmf construct. A 1:1 ratio of Bcl-2 transfected cells to Bmf transfected cells are fixed, permeabilized and contacted with the immunointeractive molecule of interest, such as a hybridoma supernatant. Visualization of antibodies bound intracellular molecules can be achieved *via* a number of techniques known to those skilled in the art, including, for example, the use of fluorescently labelled reporter molecules. Where the antibody of interest is directed to Bmf, a double

fluorescence peak is observed, the lower intensity peak representing background staining of the Bcl-2 transfected negative control cells.

In another aspect of the present invention, the molecules of the present invention are also useful as screening targets for use in applications such as the diagnosis of disorders which are regulated by Bmf. For example, screening for the levels of Bmf or *bmf* in tissue as an indicator of a predisposition to, or the development or, cancer, a degenerative disease or infertility. The screening of this aspect of the present invention may also be directed to detecting mutations in Bmf or *bmf*.

Accordingly, another aspect of the present invention contemplates a method for detecting Bmf in a biological sample from a subject, said method comprising contacting said biological sample with an immunointeractive molecule as hereinbefore defined specific for Bmf or its derivatives thereof for a time and under conditions sufficient for an immunointeractive molecule-Bmf complex to form, and then detecting said complex.

Preferably said immunointeractive molecule is an antibody. Even more preferably, said antibody is a monoclonal antibody.

Reference to biological sample according to this aspect of the present invention should be understood as a reference to any sample comprising tissue from a subject, said "tissue" should be understood in its broadest sense to include biological fluid, biopsy samples or any other form of tissue or fluid or extracts therefrom such as DNA or RNA properties.

Still another aspect of the present invention contemplates a method for detecting *bmf* in a biological sample from a subject, said method comprising contacting said biological sample with an immunointeractive molecule as hereinbefore defined specific for *bmf* or its derivatives thereof for a time and under conditions sufficient for an immunointeractive molecule-*bmf* complex to form, and then detecting said complex.

Reference to an "immunointeractive" molecule should be understood as a reference to any molecule which couples, binds or otherwise associates with *bmf* or Bmf or derivative thereof. For example, said interactive molecule may be a nucleic acid molecule or an anti-nuclear antibody.

The presence of Bmf may be determined in a number of ways such as by Western blotting, ELISA or flow cytometry procedures. *Bmf* mRNA or DNA may be detected, for example, by *in situ* hybridization or Northern blotting or Southern blotting. These, of course, include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. These assays also include direct binding of a labelled antibody to a target.

Sandwich assays are among the most useful and commonly used assays and are favoured for use in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may either be qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of hapten. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including any minor variations as will be readily apparent. In accordance with the present invention the sample is one which might contain Bmf including cell extract, tissue biopsy or possibly serum, saliva, mucosal secretions, lymph, tissue fluid and respiratory fluid. The sample is, therefore, generally a biological sample comprising

biological fluid but also extends to fermentation fluid and supernatant fluid such as from a cell culture.

In the typical forward sandwich assay, a first antibody having specificity for the Bmf or antigenic parts thereof, is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes or overnight if more convenient) and under suitable conditions (e.g. from room temperature to about 40°C such as 25°C) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and dried and incubated with a second antibody specific for a portion of the hapten. The second antibody is linked to a reporter molecule which is used to indicate the binding of the second antibody to the hapten.

An alternative method involves immobilizing the target molecules in the biological sample and then exposing the immobilized target to specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most

commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, β-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable color change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample. "Reporter molecule" also extends to use of cell agglutination or inhibition of agglutination such as red blood cells on latex beads, and the like.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic color visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method.

However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

The present invention also contemplates genetic assays such as involving PCR analysis to detect *bmf* or its derivatives.

The present invention further provides genetically modified animals in which one or both alleles of *bmf* are mutated alone or in combination with another mutation in one or both alleles for another *Bcl-2* molecule such as but not limited to genes encoding Blk, Bad, Bik, Hrk, Bid, Bim, Noxa, blx3 and/or Puma. The animals may also have mutations in other genes or alleles of genes. Preferably, the genetically modified animals are laboratory test animals such as murine species (e.g. mice, rats), rabbits, guinea pigs or hamsters, livestock animals such as sheep, pigs, horses or cows or non-human mammals such as primates. Conveniently, and preferably, the genetically modified animal is a murine species such as a mouse or rat.

The genetic modification is generally in the form of a mutation such as a single or multiple nucleotide substitution, deletion and/or addition or inversion or insertion. Generally, such a genetically modified animal is referred to as a "knock-out" animal.

Genetically modified animals and in particular knock-out murine animals may be prepared by any number of means. In one method, a targeting DNA construct is prepared comprising a nucleotide sequence which is sufficiently homologous to a target sequence such a *bmf* or *bim* to permit homologous recombination. The *bmf* or *bim* targeting sequence may be isogenic or non-isogenic to the target Bmf or Bim sequence. The targeting DNA construct generally comprises a selectable marker within the targeting sequence such that by homologous recombination, the target *bmf* or *bim* gene is disrupted by an insertional mutation. The targeting DNA construct is generally introduced into an embryonic stem cell or embryonic stem cell line. One suitable targeting vector is shown in Figure 5A.

As an alternative to using a selectable marker, a mutation may be introduced which induces a phenotypic change which may then be selected or detected.

Accordingly, another aspect of the present invention provides a method of producing a genetically modified non-human animal, said method comprising introducing into embryonic stem cells of an animal a genetic construct comprising a *bmf* nucleotide sequence carrying a single or multiple nucleotide substitution, addition and/or deletion or inversion or insertion wherein there is sufficient *bmf* nucleotide sequences to promote homologous recombination with a *bmf* gene within the genome of said embryonic stem cells selecting for said homologous recombination and selecting embryonic stem cells which carry a mutated *bmf* gene and then generating a genetically modified animal from said embryonic stem cell.

Preferably, the genetically modified animal is a murine species such as a mouse or rat.

The *Bmf* nucleotide sequence may be isogenic or non-isogenic to the *bmf* gene in the embryonic stem cell.

The term "isogenic" means that the *bmf* nucleotide sequence in the construct is derived from the same animal strain from which the embryonic stem cell has been derived.

The present invention further contemplates non-homologous-mediated integration of the target DNA sequence.

A range of selectable markers may be employed and reference may be made to U.S. Patent No. 5,789,215 for general methodologies.

The above method may be similarly adopted for introducing a plurality of mutations into different genes such as, in addition to *bmf*, other Bcl-2 genes (e.g. those encoding Bim, Blk, Bad, Bid, Hrk, Noxa or Puma) and/or other structural or regulatory genes.

Breeding protocols may also be adopted to introduce mutations or other genetic modifications into Bmf. In one approach, an EMS or other mutagenized mouse is crossed with a non-mutagenized mouse to produce a G1 generation. The G1 generation may then be crossed with an index strain to produce GIFI kindreds which are then screened phenotypically for mutation in *bmf*. Mutations in *bmf* may be dominant or recessive and mutations may be detected directly on *bmf* or by its effect on another gene or on its effect in alleviating the effects of a first mutation on another gene.

All genetically modified animals including knock-out mice carrying mutations in one or both *bmf* alleles alone or in combination with mutations in other genes such as other Bcl-2 family genes are encompassed by the present invention.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1

Identification and cloning of Bmf

The inventors sought novel BH3-only proteins that played a role in embryogenesis. Since Mcl-1--deficient mice have the most severe developmental defect of all knock-out mice lacking pro-survival Bcl-2 family members, Mcl-1was used as bait. Bmf (Bcl-2 modifying factor) was identified through yeast 2-hybrid screening of a day 17 mouse embryonic library using Mcl-1 as bait. The method used is as follows.

The cDNA libraries from day 17 mouse embryos or from mouse embryos from embryonic day 9 to one day post-partum were prepared in pAD-GAL4-2.1 (HybriZAP-2.1 kit, Stratagene). The bait vector was made by cloning mouse *mcl*-1 lacking the sequences encoding its hydrophobic C-terminus into pGBT-9 (Clontech). Yeast transformation and plasmid rescue were performed as previously described (Puthalakath *et al.*, 1999, *supra*). 7 x 10^5 clones were screened and one positive clone was obtained. Interaction between Mcl-1 and the novel protein was confirmed by β-galactosidase staining (Puthalakath *et al.*, 1999, *supra*). Sequence analysis revealed that the clone was a partial one lacking the 5' end. This partial clone was used as the probe to isolate full-length clones by screening a cDNA library derived from the $p53^{-/-}$ KO52DA20 thymoma cell line (Strasser *et al., Cell 79:* 329, 1994). Human *bmf* was isolated by screening a human activated T cell cDNA library using mouse *bmf* as probe. To screen for Bmf-interacting proteins, mouse *bmf* was subcloned into a pGBT-9 derivative harboring the gene for chloramphenicol acetyltransferase as the selection marker. Out of 5 x 10^6 clones screened, 60 positive clones were initially selected, of which 6 were later found to be false positives.

Detailed sequence analysis (Krogh *et al.*, 1994, *supra*) revealed that Bmf harbors a BH3 domain most similar to that found in Bim, Bik and EGL-1 (Figures 1A and B). In the yeast 2-hybrid system, Bmf interacted with Mcl-1 and other pro-survival Bcl-2 proteins (Bcl-2, Bcl-x_L and Bcl-w) but not with the pro-apoptotic family members tested (Bax, Bid and Bad). When transiently overexpressed in 293T cells, Bmf could be co-immunoprecipitated with pro-survival Bcl-2 family members Bcl-2 and Bcl-w (Figure 1C), as well as Bcl-x_L and Mcl-

1, but did not bind pro-apoptotic Bax or the BH3-only protein Bim. The interaction of Bmf with Bcl-2 or Bcl-w was greatly diminished by mutating the invariant leucine (L138A) within its BH3 domain (Figure 1C). Furthermore, mutations of conserved residues within the BH1 (G145E) or BH2 (W188A) domain of Bcl-2, which abolish its binding to Bim (O'Connor *et al., EMBO J. 17:* 384, 1998) or Bax (Yin *et al., Nature 369:* 321, 1994), also disrupt its binding to Bmf. Significantly, endogenous Bmf could be co-immunoprecipitated with endogenous Bcl-2 from detergent lysed MCF-7 human breast carcinoma cells (Figure 1D), excluding the possibility that these proteins associate only when overexpressed.

The biological activity of Bmf was investigated by transiently overexpressing it in Jurkat human T lymphoma cells, as well as in stably transfected L929 mouse fibroblasts (Figure 1E) or in IL-3-dependent FDC-P1 mouse promyelocytic cells (Figure 2C). Expression of Bmf triggered apoptosis in ~80% of Jurkat cells within 24 hours and reduced formation of L929 fibroblast colonies by about 65% (Figure 1E). Bmf-induced apoptosis in Jurkat cells could be blocked by the caspase inhibitor baculovirus p35, or by co-expression of Bcl-2 or its homologs (Bcl-x_L, Bcl-w, Mcl-1) but not by BH1 (G145E) or BH2 (W188A) domain mutants of Bcl-2. Consistent with its pro-apoptotic activity, high levels of Bmf could be expressed stably in FDC-P1 cells only when Bcl-2 (or one of its homologs) was also expressed. Such Bmf/Bcl-2 co-expressing FDC-P1 cells died more rapidly than Bcl-2 expressing cells in response to cytokine withdrawal (Figure 2C), γ-irradiation or treatment with etoposide. In all the cell death assays performed, Bmf mutants that lack the BH3 domain or have the L138A mutation in it were inert (Figures 1E and 2C). These results establish that Bmf is a BH3-only protein that binds pro-survival Bcl-2 family members to initiate apoptosis.

EXAMPLE 2

Expression patterns of Bmf

The expression pattern of Bmf was investigated by Northern blotting, RT-PCR and Western blotting. *bmf* mRNA was found in many cell lines of B and T lymphoid, myeloid or fibroblastoid origin and in mouse embryos at all developmental stages from E9 to birth

(Figure 1F). Western blotting of cell lysates using affinity purified rabbit polyclonal antibodies or rat monoclonal antibodies (described below) detected a single band corresponding to Bmf in many organs, with prominent levels found in pancreas, liver, kidney and hematopoietic tissues (Figure 1G). Thus, Bmf is expressed during embryogenesis and in many adult tissues.

Monoclonal rat antibodies to dynein light chains and Bmf were generated using a previously published protocol (O'Reilly *et al.*, 1998, *supra*). In brief, Wistar rats were immunized with purified recombinant mouse DLC1/LC8 or mouse Bmf. Spleen cells from immunized rats were fused with Sp2/0 myeloma cells. The resulting hybridoma clones were screened for production of specific antibodies by immunofluorescent staining and flow cytometric analyses. Hybridomas were cloned twice and antibodies were purified either on a protein-G column (Amersham Pharmacia) or on a sepharose column conjugated with MAR 18.5 (monoclonal mouse anti-rat Igκ) antibodies. Monoclonal antibody 11F7 (rat IgG 2a/κ) recognizes mouse and human DLC1/LC8 and DLC2 whereas 10D6 (rat μ/κ) detects mouse and human DLC1/LC8 but not DLC2. Monoclonal antibodies 9G10 and 12E10 (both rat γ2a/κ) detect endogenous mouse and human Bmf by Western blotting and immunoprecipitation. To generate polyclonal anti-Bmf antibodies, New Zealand White rabbits were immunized with 500 μg of recombinant mouse Bmf. Booster immunisations were given at intervals of three weeks. Serum was collected after 12 days and purified over a sepharose column conjugated with recombinant mouse Bmf protein.

EXAMPLE 3

Apoptotic structure

To assess whether *bmf* expression was induced by apoptotic stimuli, RT-PCR analyses were performed of mRNA from thymocytes exposed to various forms of stress, including cytokine deprivation, γ-irradiation or treatment with dexamethasone or ionomycin (described below). None of these stimuli had any impact on *bmf* expression (Figure 2A), prompting the inventors to investigate whether Bmf is regulated post-translationally, perhaps by interacting with other proteins. A yeast 2-hybrid screen of a mouse embryo

cDNA library with Bmf as bait isolated 14 independent clones of Mcl-1 and, surprisingly, more than 40 clones encoding dynein light chain (DLC). In a previous screen, Bim had isolated exclusively DLC1/LC8 (Puthalakath *et al.*, 1999, *supra*). In contrast, most dynein light chain clones interacting with Bmf encoded the closely related protein DLC2 (Naisbitt *et al.*, *J. Neurosci. 20:* 4524, 2000). Co-immunoprecipitation experiments in transiently transfected 293T cells confirmed the interaction of Bmf with DLC2 (Figure 2B). Sequence comparison revealed that Bmf has, in addition to the BH3 domain, a short region (aa67-DKATQTLSP) that closely resembles one in Bim (aa51-DKSTQTPSP) that mediates its binding to DLC1/LC8 (Figure 1A). This is the DLC-binding motif of Bmf, because mutations within it (A69P or D67K68A69>AAA, hereafter referred to as AAA mutation) abrogated the interaction of Bmf with DLC2 in yeast and in mammalian cells (Figure 2B). Moreover, upon IL-3 deprivation or γ-irradiation, FDC-P1 cells co-expressing Bcl-2 and non-DLC2-binding mutants of Bmf died much more rapidly than those co-expressing Bcl-2 and wild-type Bmf (Figure 2C). These Bmf mutants also suppressed the formation of L929 fibroblast colonies more potently than wild-type Bmf. Hence, interaction with DLC2 negatively regulates the pro-apoptotic activity of Bmf.

RT-PCR analysis of *bmf* mRNA expression was performed using the following primers: 5' (sense) primer 5'CCGGATGGATCACCAGGAATG3' [SEQ ID NO:11], 3' (antisense) primer 5'CAGAGCTGACAAAGGCACAG3' [SEQ ID NO:12]. Detection of the PCR products on Southern blots was performed using the internal *bmf* primer 5'CCACTTCCTGGAGAACATCA3' [SEQ ID NO:13]. For analysis of GAPDH expression, the following primers were used: 5' (sense) primer 5'TGATGACATCAAGAAGGTGGTGAAG3' [SEQ ID NO:14], 3' (antisense) primer 5'TCCTTGGAGGCCATGTAGGCCAT3' [SEQ ID NO:15] and the internal primer 5'CCCGGCATCGAAGGTGGAAGAG3' [SEQ ID NO:16].

EXAMPLE 4

Functional model

The question considered by the inventors is why Bmf is controlled by binding to highly related partners, DLC-1 or DLC-2. It is proposed that Bmf is sequestered to sites within the cell in order to sense distinct stress stimuli. Separation of cellular proteins into the filamentous actin and the paclitaxel (taxol)-polymerizable microtubular fractions revealed that, consistent with previous results (Puthalakath *et al.*,1999, *supra*), Bim and dynein intermediate chain (IC74) largely co-migrated with microtubular components (P2), whereas Bmf and myosin V were confined to the filamentous actin-containing P1 fraction (Figure 3A). Furthermore, treating cells with actin depolymerizing agents, such as cytochalasin D or *C. difficcili* toxin B, released Bmf from the filamentous actin-containing P1 fraction whereas the fractionation of Bim was unaffected (Figure 3B).

For subcellular fractionation, 5 x 10^6 MCF-7 cells were lysed in 500 µL extraction buffer containing 1% Triton-X-100. Cell debris and nuclei were removed by centrifugation at 2000 *g*. The supernatant was then incubated for 13 minutes at 37°C with 100 µM paclitaxel (taxol) and 5 units of apyrase (Sigma). This mixture was then loaded on top of a 0.5 mL cushion of 7.5% sucrose (made in the extraction buffer) and centrifuged at 140,000 *g* for 30 minutes at 30°C. The pellet was saved as the microtubular P2 fraction and the supernatant as the S fraction. To obtain the actin-enriched P1 fraction without contamination by microtubular constituents, MCF-7 cells were cultured for 2 hrs in the presence of 2 µg/mL colchicine and 1 µg/mL nocodazole prior to lysis. These lysates were then cleared of cell debris and nuclei (described above) and subsequently centrifuged for 60 minutes at 4°C at 140,000 *g* to obtain the pellet (P1) fraction. For fractionation of extracts on sucrose gradients, 10^7 cells were lysed in 500 µL extraction buffer. After removing cellular debris and nuclei, the supernatants were treated with 100 µM paclitaxel (taxol) plus 5 units of apyrase and incubated at 37°C for 13 minutes before loading onto a 5-20% sucrose gradient (prepared in extraction buffer containing 1% Triton X-100) and centrifuging for 18 hours at 15°C at 140,000 *g*.

The distinct localization of Bmf and Bim may be determined largely by their preferred dynein light chain partners. Contrary to a previous report (Benashski *et al., J. Biol. Chem. 272:* 20929, 1997), by using monoclonal antibodies that either recognize only DLC1/LC8 or both DLC1/LC8 and DLC2 (Figure 3C), the inventors showed that purified myosin V motor complexes contained DLC2 but not DLC1/LC8 (Figure 3D). This observation indicated that Bmf, by being preferentially bound to DLC2, might be complexed with myosin V on filamentous actin rather than forming part of the dynein motor complex. Consistent with this notion, incubation of extracts from mouse spleen cells with recombinant Bmf and Bim confirmed that only Bmf associated with myosin V (Figure 3E). Furthermore, Bmf and Bim showed distinct migration patterns after subcellular fractionation of lysates from MCF-7 cells on sucrose gradients (Figure 3F). Since DLC1/LC8 forms homodimers avidly and since it binds Bim and IC74 through the same region (Lo *et al., J. Biol. Chem. 276:* 14059, 2001), one partner of a DLC1/LC8 homodimer probably interacts with IC74 whilst the other binds Bim, thereby sequestering it to the microtubular dynein motor complex. It is likely that DLC2 homodimers sequester Bmf to filamentous actin by binding with one arm to Bmf and with the other to myosin V.

The inventors next investigated whether Bmf and Bim are activated by distinct apoptotic stimuli using cells that express both proteins endogenously. Consistent with our previous results (Puthalakath *et al.*,1999, *supra*), UV-irradiation of MCF-7 cells released Bim from the pellet fraction where the dynein motor complex resided. When lysates of healthy or damaged MCF-7 cells were compared by sucrose gradient centrifugation, it became apparent that Bmf also translocated from denser to lighter fractions in response to UV-irradiation (Figure 4A). Treatment with paclitaxel (taxol), a chemotherapeutic drug known to polymerize microtubules, released Bim but not Bmf (Figure 4A). Consistent with a critical role for Bim in this pathway to apoptosis, Bim-deficient thymocytes are abnormally resistant to the cytotoxic effects of paclitaxel (Frisch and Ruoslahti, *Science 286(5445):* 1735-1738, 1999). On the other hand, anoikis (absence of cell attachment and integrin signaling), an apoptotic stimulus that affects the actin cytoskeleton (Frisch and Ruoslahti, 1997, *supra*), resulted in the selective release of Bmf but not Bim (Figure 4A). Since these experiments were conducted in the presence of the broad-spectrum caspase inhibitor zVAD-fmk at a

concentration (50 μM) sufficient to block caspase activation, the release of Bmf and/or Bim are likely to represent initiating events in apoptosis signaling rather than being a consequence of apoptotic changes. Importantly, the inventors showed that endogenous Bmf (together with DLC2) released during anoikis could be co-immunoprecipitated with endogenous Bcl-2 isolated from mitochondria (Figure 4B). In contrast, negligible Bmf was found complexed with Bcl-2 isolated from mitochondria of healthy cells.

Collectively, the inventors' data demonstrate that Bmf and Bim represent two pro-apoptotic BH3-only proteins that transduce distinct death signals caused by different forms of cell stress. They seem to represent sentinels mounted on the main cytoskeletal structures to monitor the well-being of the cell. For example, disturbance of the microtubules by paclitaxel activates Bim but not Bmf, whereas anoikis, which affects the actin cytoskeleton, activates Bmf but not Bim. Since deregulated expression of anti-apoptotic Bcl-2 can promote tumorigenesis (Strasser *et al., Nature 348:* 331, 1990), it is possible that abnormalities in pro-apoptotic BH3-only proteins can also cause cancer. The gene for human *bmf* is located on chromosome 15q14, identified as the site of a candidate tumor suppressor gene lost in many metastastic but not primary carcinomas (Wick *et al., Oncogene 12:* 973, 1996). Anoikis has been implicated as a barrier against metastatic tumor growth (Ruoslahti and Reed, *Cell 77:* 477, 1994). Metastatic tumors harboring 15q14 mutations may, therefore, have abnormalities in their expression or function of Bmf.

EXAMPLE 5

Generation of Bmf knock-out mice

Mice are selected with a C57BL/6 background which are back crossed into C57BL/6. Offspring are genotyped using PCR using primers specific for wild-type or mutant *bmf* genes.

A *bmf* targeting vector is generated as shown in Figure 5A. A neomycin or hygromycin sequence is used as the selectable marker. The construct is introduced into embryonic stem

cells and transformed cells selected using neomycin or hygromycin. The transformed embryonic stem cells are then used to generate genetically modified mice.

EXAMPLE 6

Genomic organization of Bmf and identification of promoter regions

The genomic organization of the murine *bmf* gene is shown in Figure 5A. Upstream of this region comprises a promoter region as outlined in SEQ ID NO:9. A corresponding promoter from the human *bmf* gene is outlined in SEQ ID NO:10.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

CLAIMS

1. A nucleic acid molecule comprising a nucleotide sequence encoding or complementary to a sequence encoding an amino acid sequence substantially as set forth in one of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or a derivative or homolog thereof or having at least about 45% or greater similarity to one or more of SEQ ID NO:2 or SEQ ID NO:4 or SEQ ID NO:6 or SEQ ID NO:8 or a derivative or homolog thereof.

2. The nucleic acid molecule of Claim 1 comprising a nucleotide sequence which encodes the amino acid sequence set forth in SEQ ID NO:2.

3. The nucleic acid molecule of Claim 1 comprising a nucleotide sequence which encodes the amino acid sequence set forth in SEQ ID NO:4.

4. The nucleic acid molecule of Claim 1 comprising a nucleotide sequence which encodes the amino acid sequence set forth in SEQ ID NO:6.

5. The nucleic acid molecule of Claim 1 comprising a nucleotide sequence which encodes the amino acid sequence set forth in SEQ ID NO:8.

6. The nucleic acid molecule of Claim 1 comprising a nucleotide sequence set forth in SEQ ID NO:1 or a nucleotide sequence having at least about 45% similarity thereto or a nucleotide sequence capable of hybridizing to SEQ ID NO:1 or its complementary form under low stringency conditions.

7. The nucleic acid molecule of Claim 1 comprising a nucleotide sequence set forth in SEQ ID NO:3 or a nucleotide sequence having at least about 45% similarity thereto or a nucleotide sequence capable of hybridizing to SEQ ID NO:3 or its complementary form under low stringency conditions.

8. The nucleic acid molecule of Claim 1 comprising a nucleotide sequence set forth in SEQ ID NO:5 or a nucleotide sequence having at least about 45% similarity thereto or a nucleotide sequence capable of hybridizing to SEQ ID NO:5 or its complementary form under low stringency conditions.

9. The nucleic acid molecule of Claim 1 comprising a nucleotide sequence set forth in SEQ ID NO:7 or a nucleotide sequence having at least about 45% similarity thereto or a nucleotide sequence capable of hybridizing to SEQ ID NO:7 or its complementary form under low stringency conditions.

10. The nucleic acid molecule of Claim 1 comprising the nucleotide sequence set forth in SEQ ID NO:1.

11. The nucleic acid molecule of Claim 1 comprising the nucleotide sequence set forth in SEQ ID NO:3.

12. The nucleic acid molecule of Claim 1 comprising the nucleotide sequence set forth in SEQ ID NO:5.

13. The nucleic acid molecule of Claim 1 comprising the nucleotide sequence set forth in SEQ ID NO:7.

14. An isolated protein comprising an amino acid sequence encoded by a nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or a nucleotide sequence having at least about 45% similarity to the nucleotide sequence set forth in SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or a nucleotide sequence capable of hybridizing to SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:5 or SEQ ID NO:7 or a complement thereof under low stringency conditions.

15. The isolated protein of Claim 14 comprising an amino acid sequence encoded by the nucleotide sequence set forth in SEQ ID NO:1.

16. The isolated protein of Claim 14 comprising an amino acid sequence encoded by the nucleotide sequence set forth in SEQ ID NO:3.

17. The isolated protein of Claim 14 comprising an amino acid sequence encoded by the nucleotide sequence set forth in SEQ ID NO:5.

18. The isolated protein of Claim 14 comprising an amino acid sequence encoded by the nucleotide sequence set forth in SEQ ID NO:7.

19. The isolated protein of Claim 14 comprising an amino acid sequence as set forth in SEQ ID NO:2 or having at least 45% similarity thereto.

20. The isolated protein of Claim 14 comprising an amino acid sequence as set forth in SEQ ID NO:4 or having at least 45% similarity thereto.

21. The isolated protein of Claim 14 comprising an amino acid sequence as set forth in SEQ ID NO:6 or having at least 45% similarity thereto.

22. The isolated protein of Claim 14 comprising an amino acid sequence as set forth in SEQ ID NO:8 or having at least 45% similarity thereto.

23. The isolated protein of Claim 14 having an amino acid sequence as set forth in SEQ ID NO:2.

24. The isolated protein of Claim 14 having an amino acid sequence as set forth in SEQ ID NO:4.

25. The isolated protein of Claim 14 having an amino acid sequence as set forth in SEQ ID NO:6.

26. The isolated protein of Claim 14 having an amino acid sequence as set forth in SEQ ID NO:8.

27. A variant of an isolated *bmf* nucleic acid molecule comprising one or more nucleotide mutations in said nucleic acid molecule resulting in at least one amino acid addition, substitution and/or deletion to the polypeptide encoded by said variant wherein said polypeptide cannot bind, couple or otherwise associate with a dynein light chain, such as DLC2.

28. The variant of Claim 28 wherein the mutation results in an altered amino acid sequence in the region which binds to the dynein light chain.

29. A variant of an isolated Bmf polypeptide comprising at least one amino acid addition, substitution and/or deletion wherein said polypeptide cannot bind, couple or otherwise associate with the dynein light chain.

30. A method of modulating activity of Bmf in a mammal, said method comprising administering to said mammal a modulating effective amount of an agent for a time and under conditions sufficient to increase or decrease Bmf activity.

31. A method of modulating apoptosis in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the expression of a nucleotide sequence encoding *bmf*.

32. A method of modulating apoptosis in a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of Bmf.

33. A method of treating a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to

modulate the expression of *bmf* wherein said modulation results in modulation of apoptosis.

34. A method of treating a mammal, said method comprising administering to said mammal an effective amount of an agent for a time and under conditions sufficient to modulate the activity of Bmf wherein said modulation results in modulation of apoptosis.

35. The method of Claim 30 or 31 or 32 or 33 or 34 wherein the mammal is a human.

36. A pharmaceutical composition comprising *bmf*, Bmf or derivative thereof or an agent capable of modulating *bmf* expression or Bmf activity together with one or more pharmaceutically acceptable carriers and/or diluents. *bmf*, Bmf or said agent are referred to as the active ingredients.

37. A monoclonal antibody having specificity for Bmf or *bmf* or derivative thereof.

38. A method of detecting an immunointeractive molecule, in a sample, specific for a protein of interest produced by a cell, said method comprising contacting the sample to be tested with a population of cells comprising a defined ratio of cells producing the protein of interest and cells not producing the protein of interest for a time and under conditions sufficient for immunointeractive molecules, if present in said sample, to interact with said protein of interest and the subjecting said immunointeractive molecule-protein complex to detecting means.

39. The method of Claim 38 wherein the interactive molecule is an antibody.

40. A genetically modified animal in which one or both alleles of *bmf* are mutated alone or in combination with another mutation in one or both alleles for another *Bcl-2* molecule such as but not limited to genes encoding Blk, Bad, Bik, Hrk, Bid, Bim, Noxa and/or Puma.

41. The genetically modified animal of Claim 40 wherein said animal is a mouse.

42. The genetically modified animal of Claim 40 wherein said animal is a rat.

43. The genetically modified animal of Claim 40 wherein said animal is a pig.

44. A method of producing a genetically modified non-human animal, said method comprising introducing into embryonic stem cells of an animal a genetic construct comprising a *bmf* nucleotide sequence carrying a single or multiple nucleotide substitution, addition and/or deletion or inversion or insertion wherein there is sufficient *bmf* nucleotide sequences to promote homologous recombination with a *bmf* gene within the genome of said embryonic stem cells selecting for said homologous recombination and selecting embryonic stem cells which carry a mutated *bmf* gene and then generating a genetically modified animal from said embryonic stem cell.

45. The method of Claim 44 wherein the genetically modified animal is a mouse or rat.

46. Use of Bmf in the manufacture of a medicament for the treatment of a condition in a human.

47. Use of Bmf in the manufacture of a medicament for the treatment of a condition in a non-human.

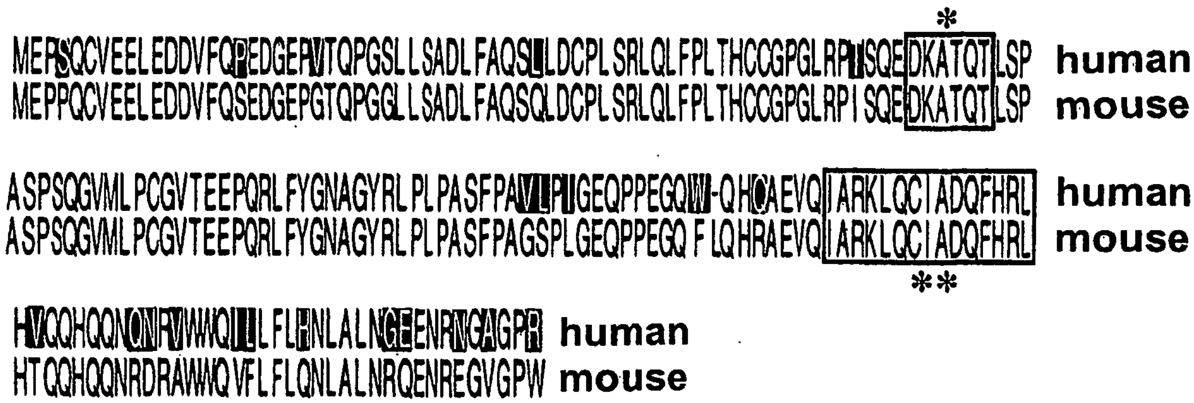


Figure 1A

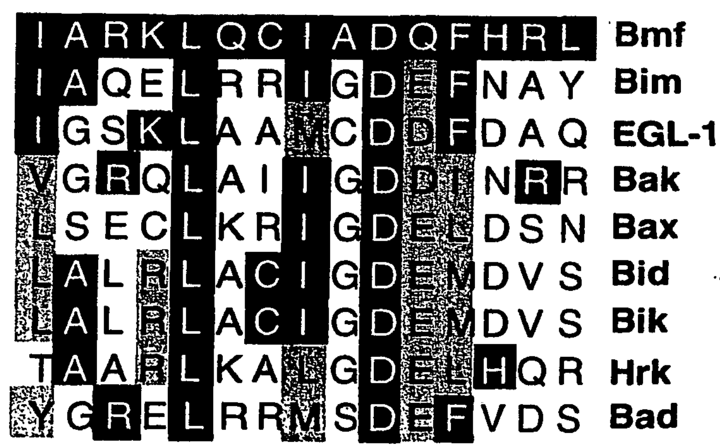


Figure 1B

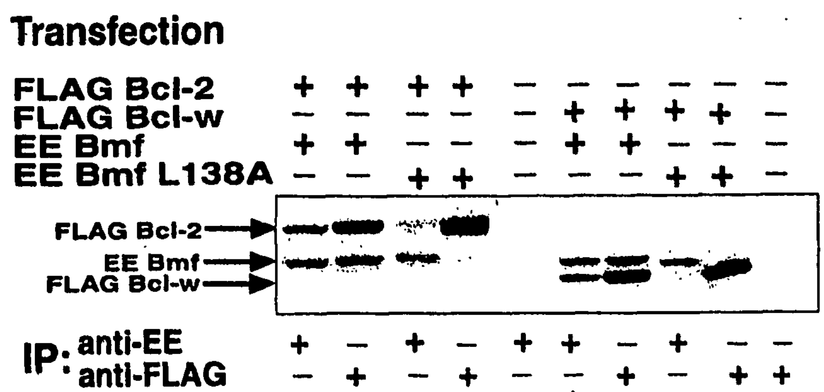


Figure 1C

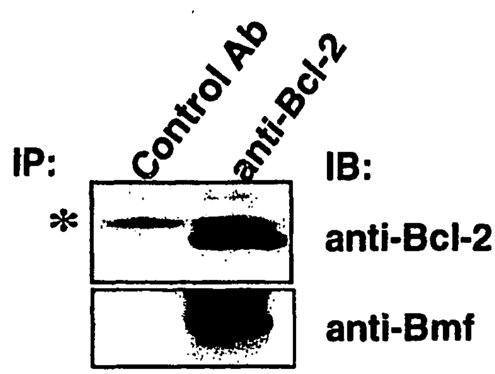


Figure 1D

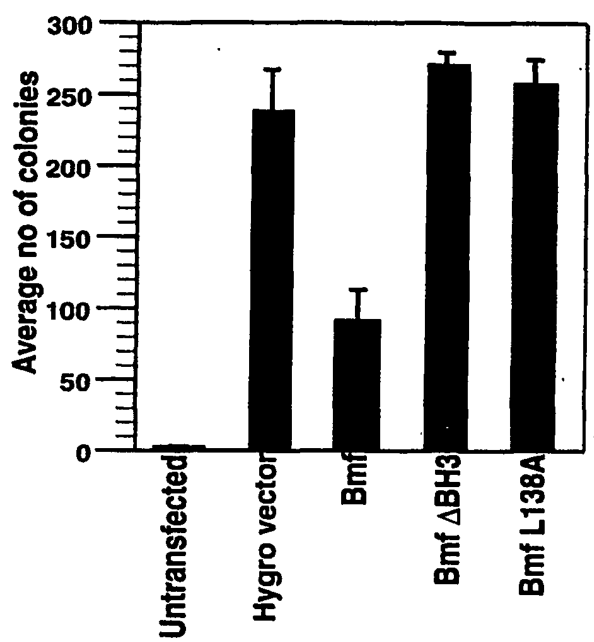

300
250
200
150
100
50
0
Average no of colonies
Untransfected
Hygro vector
Bmf
Bmf ΔBH3
Bmf L138A

Figure 1E

Figure 1F

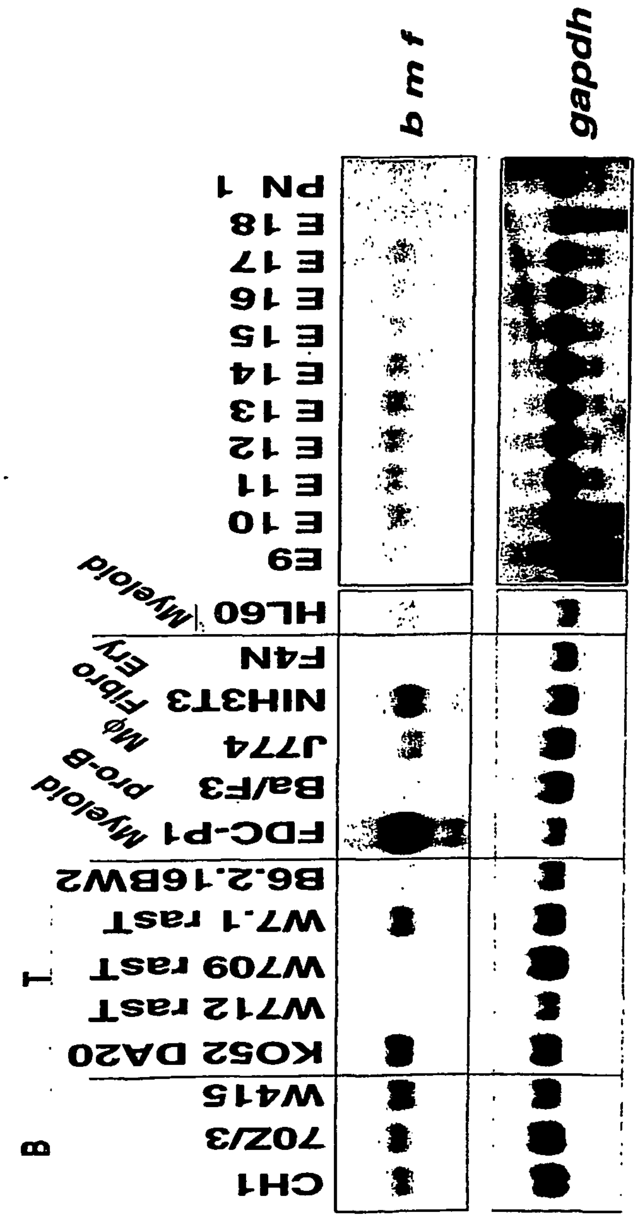
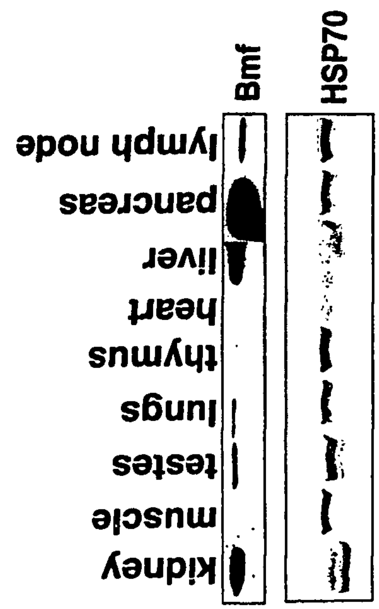


Figure 1G



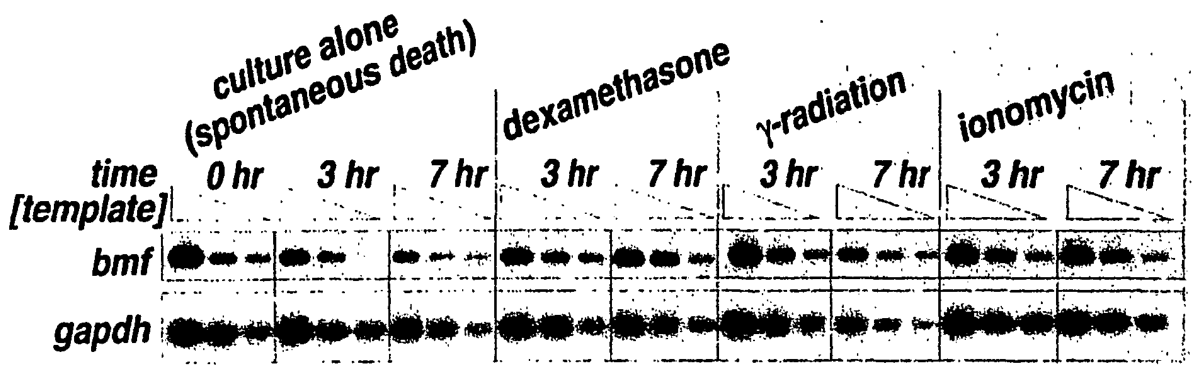


Figure 2A

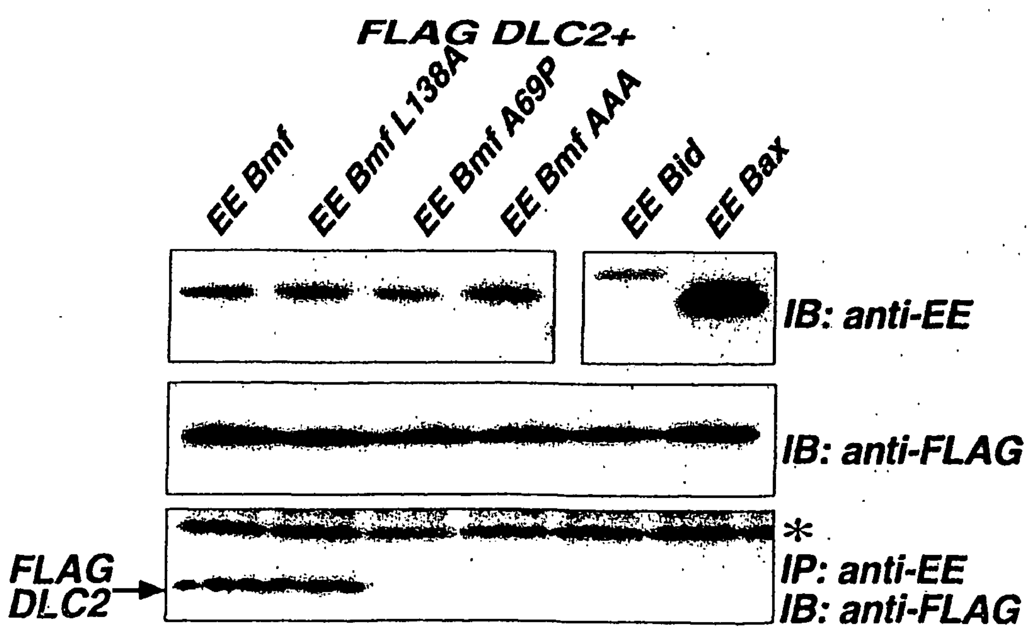


Figure 2B

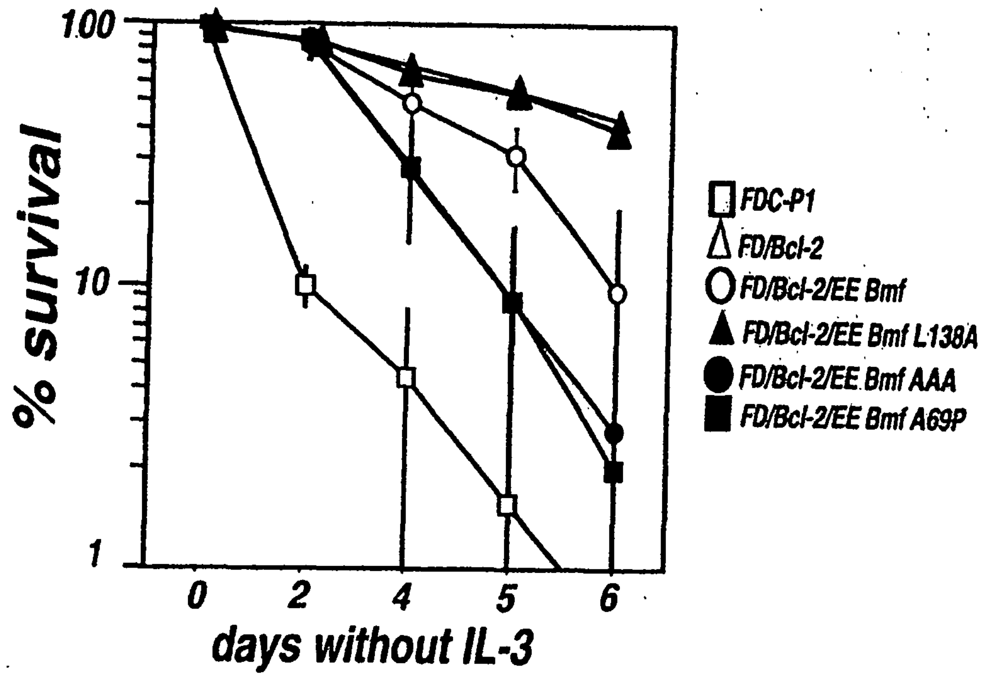

100
10
1
% survival
0
2
4
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Figure 2C

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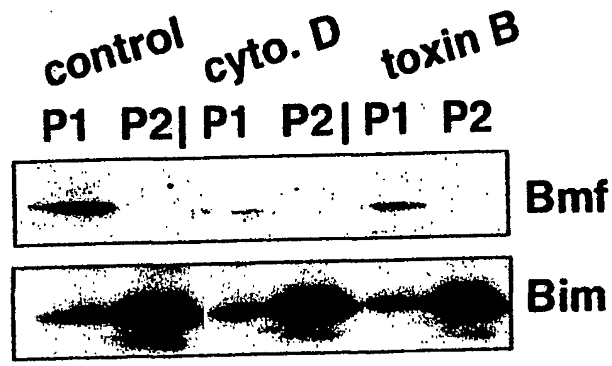

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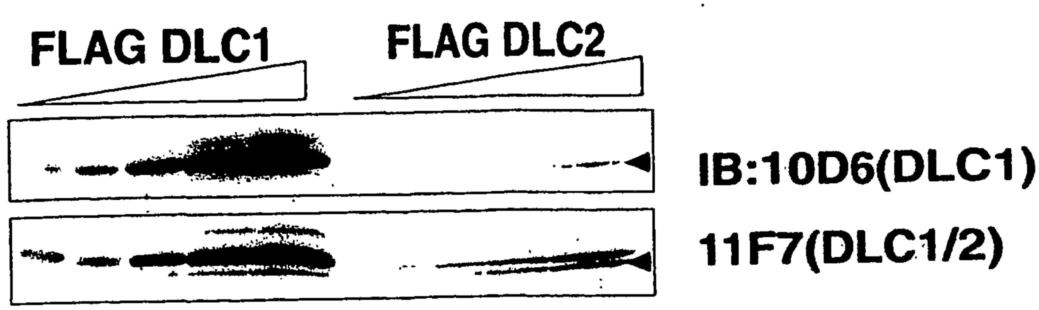

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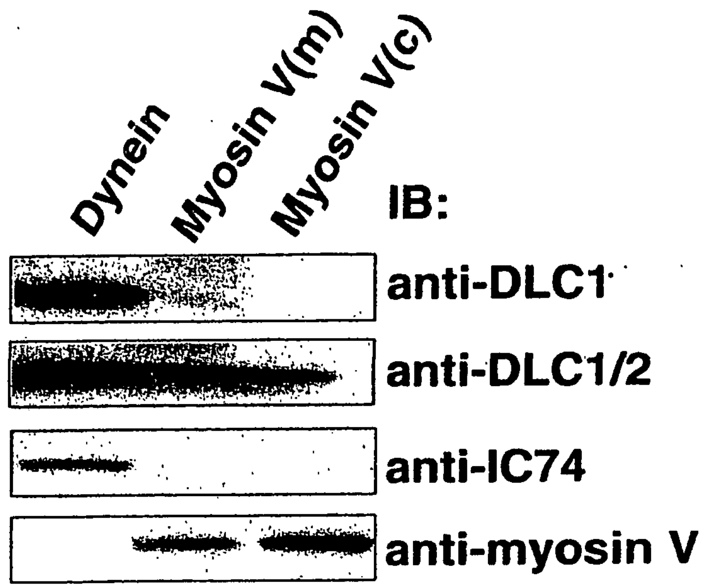

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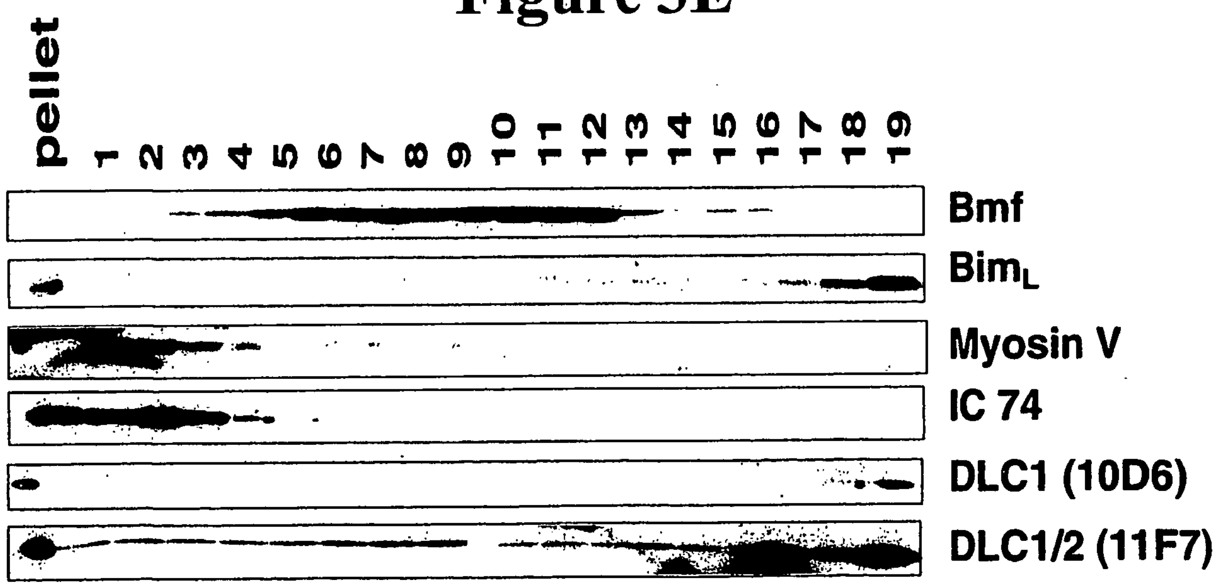

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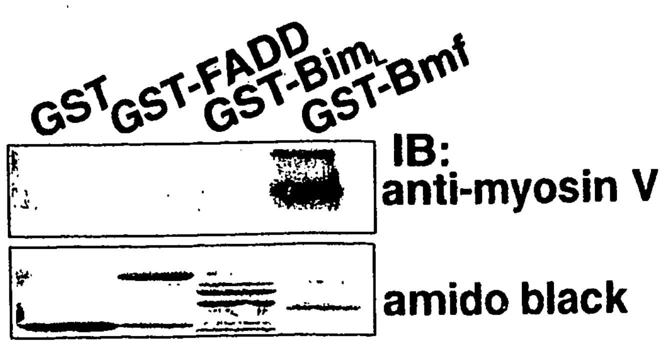

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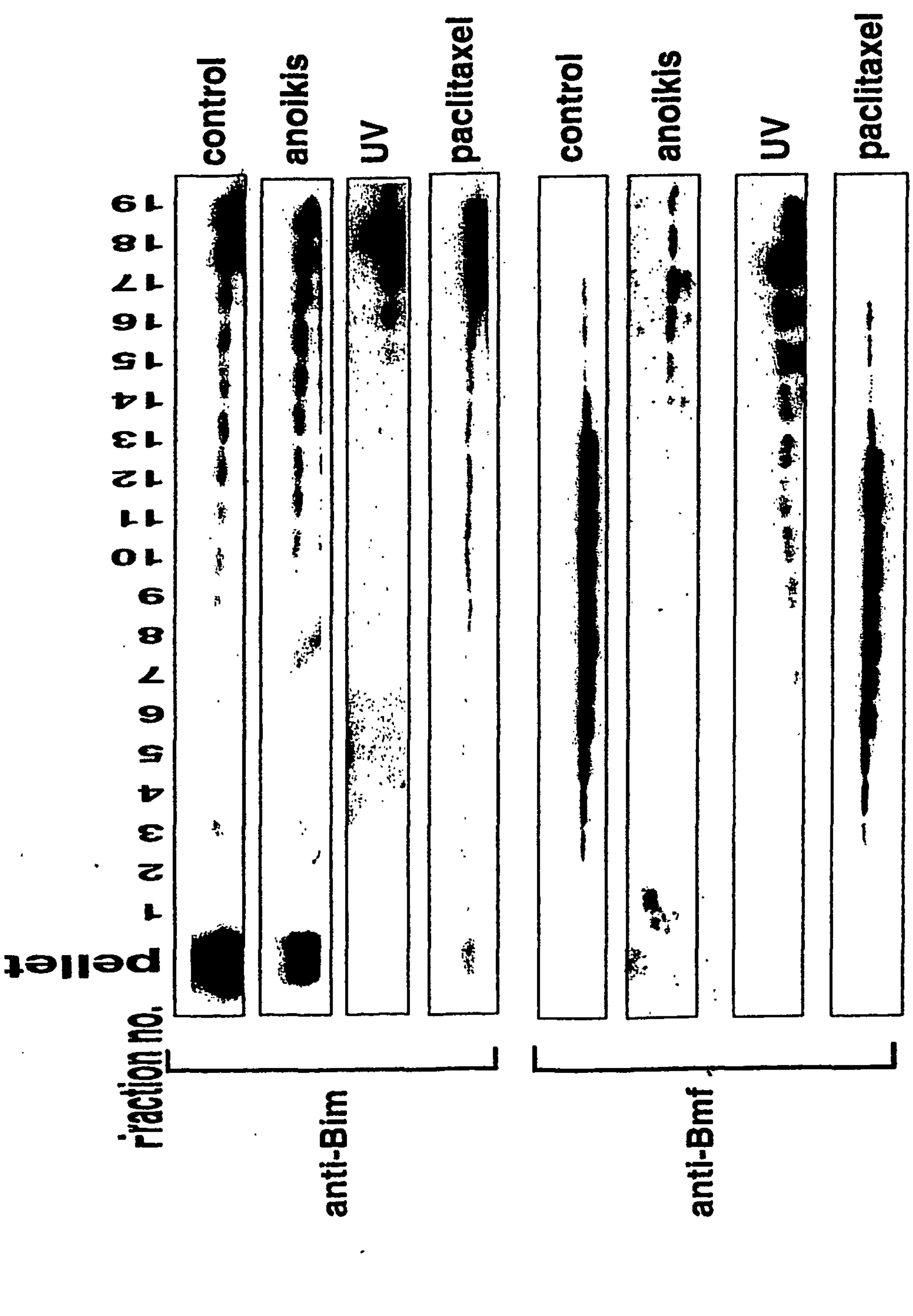
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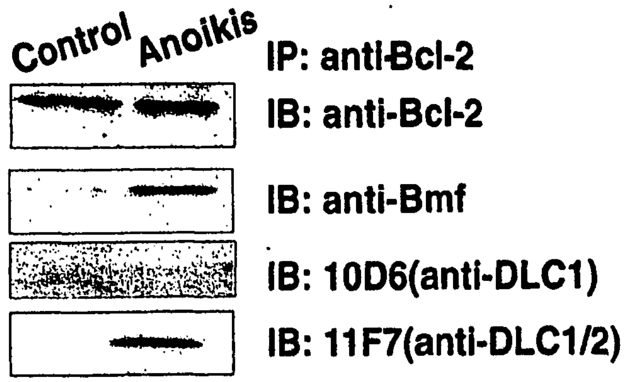
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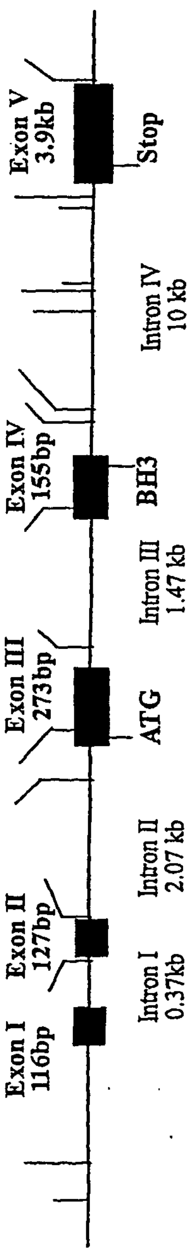
Figure 4B



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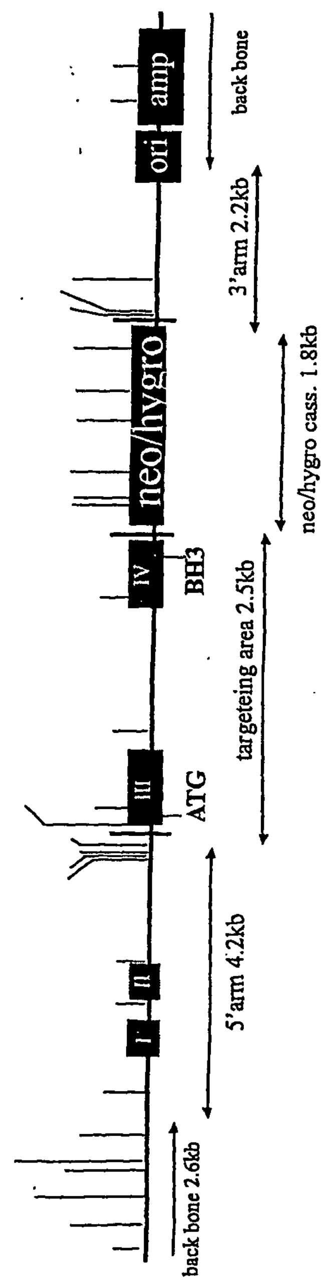

Figure 5B

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<400> 7
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<210> 8
<211> 18
<212> PRT
<213> mouse

<220>
<221> misc_feature
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<223> BH3 doman of human bmf

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1 5 10 15

His Val

<210> 9
<211> 2000
<212> DNA
<213> mouse

<220>
<221> misc_feature
<222> ()..()
<223> bmf promoter

<400> 9

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ttctagcaac cacagccctc aatatggctg gttcattctg tttcaggtga cttctgaggg    240
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ggtcccagct ccactgaact taaatctaag tttacaaccc agagagcagc tcggtggtca    360
gagcactgct gagccctact gcccctggtg ggtcagggtt gttgcaggac tggctccctc    420
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ggcgacggcg ctccgactgc gcttctggcg acggtcggaa ttttgctcgg ccccttgcaa 1860

tgtttccatg ggaaggttcg tacattcgtg accgtccctg gcagcggccc agcccgggac 1920

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<211> 1833
<212> DNA
<213> human

<220>
<221> misc_feature
<222> ()..()
<223> bmf promoter

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atgcatgaag gcaaaacacc cacacgaata aagtaaaaat aaatatatct aaaaacaatt 180

aaaaaagaaa aacagaaaga aagaaaccaa agtaaataaa taaaaatttt aaaacaaaaa 240

atagccaaag acaattgagt ctgggagccc cagatccagc cagcttgtat cttagagcag 300

actgtaagta gagactgtaa ctaagcccag cttctctgct ttgcccgtgt ctctattgca 360

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caaattactt ttaaaaaata cagactcctc agcctggtat tccagggggc acctctgcaa 720

tctgacctga atctacctgc ccataccctc acatcctaaa tcatagtgaa gatgccagag 780

agcttgatgg gggggagggg ggacgggggg ggcgtgcagc tgatcttgct ttccagacat 840

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aagtcctgca ataactcagc ccatttccaa gtagacaaaa gtcacttagg cgtcttgtcc 1560

ccccactacc tagtagaaaa cctagggact ctctgtcgtt tctggggctt gcattaggaa 1620

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<211> 21
<212> DNA
<213> artificial sequence

<220>
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<222> ()..()
<223> 5' sense primer

<400> 11
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<210> 12
<211> 19
<212> DNA
<213> artificial sequence

<220>
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<222> ()..()
<223> 3' antisense primer

<400> 12
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<210> 13
<211> 20
<212> DNA

<213> artificial sequence

<220>
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<222> ()..()
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<400> 13
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<210> 14
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<220>
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<210> 16
<211> 22
<212> DNA
<213> artificial sequence

<220>
<221> misc_feature
<222> ()..()
<223> internal primer

<400> 16
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<210> 17
<211> 185
<212> PRT
<213> mouse

<220>
<221> misc_feature
<222> ()..()

<223> predicted amino acid sequence of mouse Bmf

<400> 17
Met Glu Pro Pro Gln Cys Val Glu Glu Leu Glu Asp Asp Val Phe Gln
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Ser Glu Asp Gly Glu Pro Gly Thr Gln Pro Gly Gly Leu Leu Ser Ala
20 25 30

Asp Leu Phe Ala Gln Ser Gln Leu Asp Cys Pro Leu Ser Arg Leu Gln
35 40 45

Leu Phe Pro Leu Thr His Cys Cys Gly Pro Gly Leu Arg Pro Ile Ser
50 55 60

Gln Glu Asp Lys Ala Thr Gln Thr Leu Ser Pro Ala Ser Pro Ser Gln
65 70 75 80

Gly Val Met Leu Pro Cys Gly Val Thr Glu Glu Pro Gln Arg Leu Phe
85 90 95

Tyr Gly Asn Ala Gly Tyr Arg Leu Pro Leu Pro Ala Ser Phe Pro Ala
100 105 110

Gly Ser Pro Leu Gly Glu Gln Pro Pro Glu Gly Gln Phe Leu Gln His
115 120 125

Arg Ala Glu Val Gln Ile Ala Arg Lys Leu Gln Cys Ile Ala Asp Gln
130 135 140

Phe His Arg Leu His Thr Gln Gln His Gln Gln Asn Arg Asp Arg Ala
145 150 155 160

Trp Trp Gln Val Phe Leu Phe Leu Gln Asn Leu Ala Leu Asn Arg Gln
165 170 175

Glu Asn Arg Glu Gly Val Gly Pro Trp
180 185

<210> 18
<211> 184
<212> PRT
<213> human

<220>
<221> misc_feature
<222> ()..()
<223> predicted amino acid sequence of human Bmf

<400> 18
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1 5 10 15

Pro Glu Asp Gly Glu Pro Val Thr Gln Pro Gly Ser Leu Leu Ser Ala
20 25 30

Asp Leu Phe Ala Gln Ser Leu Leu Asp Cys Pro Leu Ser Arg Leu Gln

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        35                  40                  45

Leu Phe Pro Leu Thr His Cys Cys Gly Pro Gly Leu Arg Pro Thr Ser
    50                  55                  60

Gln Glu Asp Lys Ala Thr Gln Thr Leu Ser Pro Ala Ser Pro Ser Gln
65                  70                  75                  80

Gly Val Met Leu Pro Cys Gly Val Thr Glu Glu Pro Gln Arg Leu Phe
                85                  90                  95

Tyr Gly Asn Ala Gly Tyr Arg Leu Pro Leu Pro Ala Ser Phe Pro Ala
            100                 105                 110

Val Leu Pro Ile Gly Glu Gln Pro Pro Glu Gly Gln Trp Gln His Gln
        115                 120                 125

Ala Glu Val Gln Ile Ala Arg Lys Leu Gln Cys Ile Ala Asp Gln Phe
    130                 135                 140

His Arg Leu His Val Gln Gln His Gln Gln Asn Gln Asn Arg Val Trp
145                 150                 155                 160

Trp Gln Ile Leu Leu Phe Leu His Asn Leu Ala Leu Asn Gly Glu Glu
                165                 170                 175

Asn Arg Asn Gly Ala Gly Pro Arg
            180

<210>  19
<211>  15
<212>  PRT
<213>  mouse/human

<220>
<221>  misc_feature
<222>  ()..()
<223>  partial amino acid sequence of Bmf

<400>  19
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1               5                   10                  15

<210>  20
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<212>  PRT
<213>  mouse/human

<220>
<221>  misc_feature
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<223>  partial amino acid sequence of Bim

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1               5                   10                  15
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<211> 15
<212> PRT
<213> mouse/human

<220>
<221> misc_feature
<222> ()..()
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<400> 21
Ile Gly Ser Lys Leu Ala Ala Met Cys Asp Asp Phe Asp Ala Gln
1 5 10 15

<210> 22
<211> 15
<212> PRT
<213> mouse/human

<220>
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<222> ()..()
<223> partial amino acid sequence of Bak

<400> 22
Val Gly Arg Gln Leu Ala Ile Ile Gly Asp Asp Ile Asn Arg Arg
1 5 10 15

<210> 23
<211> 15
<212> PRT
<213> mouse/human

<220>
<221> misc_feature
<222> ()..()
<223> partial amino acid sequence of Bax

<400> 23
Leu Ser Glu Cys Leu Lys Arg Ile Gly Asp Glu Leu Asp Ser Asn
1 5 10 15

<210> 24
<211> 15
<212> PRT
<213> mouse/human

<220>
<221> misc_feature
<222> ()..()
<223> partial amino acid sequence of Bid

<400> 24
Leu Ala Leu Arg Leu Ala Cys Ile Gly Asp Glu Met Asp Val Ser
1 5 10 15

<210> 25

<211> 15
<212> PRT
<213> mouse/human

<220>
<221> misc_feature
<222> ()..()
<223> partial amino acid sequence of Bik

<400> 25
Leu Ala Leu Arg Leu Ala Cys Ile Gly Asp Glu Met Asp Val Ser
1 5 10 15

<210> 26
<211> 15
<212> PRT
<213> mouse/human

<220>
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<222> ()..()
<223> partial amino acid sequence of Hrk

<400> 26
Thr Ala Ala Arg Leu Lys Ala Leu Gly Asp Glu Leu His Gln Arg
1 5 10 15

<210> 27
<211> 15
<212> PRT
<213> mouse/human

<220>
<221> misc_feature
<222> ()..()
<223> partial amino acid sequence of Bad

<400> 27
Tyr Gly Arg Glu Leu Arg Arg Met Ser Asp Glu Phe Val Asp Ser
1 5 10 15

INTERNATIONAL SEARCH REPORT	International application No. PCT/AU02/00693

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12N 15/12, C07K 14/435, 16/18, A61K 38/17, 39/395, 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

SEE ELECTRONIC DATABASE BOX BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SEE ELECTRONIC DATABASE BOX BELOW

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

GENPEPT, SWISS-PROT, PIR, EMBL, GENBANK: SEQ ID NOs: 2, 4, 6, 8.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL database Accession Number AK024472. Homo sapiens mRNA for FLJ00065 protein. 29 September 2000. & GenPept database Accession Number BAB15762. See whole document, the sequence shows 100% homology with SEQ ID NO:4, 6 and 8 and 87% homology with SEQ ID NO: 2.	1, 3-9, 11-14, 16-22 and 24-26
X	EMBL database Accession Number AC025429. Homo sapiens chromosome 15 clone CTD-2006D8 map 15q14. 15 March 2000. See whole document, the sequence shows 100% homology with SEQ ID NO:4, 6 and 8 and 87% homology with SEQ ID NO: 2.	1, 3-9, 11-14, 16-22 and 24-26

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 July 2002	17 JUL 2002

Name and mailing address of the ISA/AU	Authorized officer
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	J.H. CHAN Telephone No : (02) 6283 2340

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU02/00693

Box I **Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos : **40 (in part)**

because they relate to subject matter not required to be searched by this Authority, namely:

Claim 40 refers to genetically modified animals. The claim does not exclude the animal from being a human being. Thus, the claim comprises excluded subject matter under Rule 39 of the PCT. Therefore this aspect of the claim has not been searched.

2. [X] Claims Nos : **38 and 39 (complete) and 36 (in part)**

because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

See additional sheet below.

3. [ ] Claims Nos :

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box II **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: I

The scope of claims 36 (in part) is unduly broad and speculative. A meaningful and economically feasible search could not encompass the complete subject matter of the claims. Claim 36 in part refers to an agent capable of modulating *bmf* expression, hence the claim encompasses an agent *per se*. There is no support for what is encompassed within the scope of the term "agent". It is not feasible to perform a meaningful and economical search on this aspect of claim 36. Therefore this aspect of claim 36 has not been searched.

Claims 38 and 39 have not been searched, as these claims do not comply with rule 6.3 of the PCT. This rule refers to the claims defining the technical features of the invention. The present invention appears to lie in the identification and characterisation of the mouse and human Bmf or BMF proteins and the nucleotides that encode them. These claims are directed to a method of detecting an immunointeractive molecule in a sample specific for a protein of interest. The claims do not comprise the mouse Bmf or human BMF proteins or the nucleotides that encode them. Thus the claims are not limited to the technical features of the invention. Therefore the claims were not searched.

C (Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	Science (2001) 293 (5536): 1829-1832. Puthalakath H *et al.* "Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis." & GenBank database accession numbers AY029253 & AY029254 & GenPept database accession numbers AAK38747 & AAK38748 respectively. See whole document, Mus musculus Bmf sequences are 100% identical to SEQ ID NOs: 1, 2, 5-8 and Homo sapiens BMF sequences are 100% identical to SEQ ID NOs: 3-8.	1-37 and 40-47
P, X	GenBank database accession number AF506761. Rattus norvegicus Bcl-2 modifying factor (Bmf), mRNA. 16 May 2002. & GenPept database accession number AAM28890. See whole document, sequence is up to 96% identical to SEQ ID NOs: 1-8.	1-37 and 40-47